INVESTIGATING THE ROLE OF THE TRANSCRIPTION FACTOR B-MYB IN NEUROBLASTOMA

Rebekka Andrea Vera Schwab

Institute of Child Health
University College London

A thesis submitted for the Degree of Doctor of Philosophy

2007

UMI Number: U593418

All rights reserved

INFORMATION TO ALL USERS
The quality of this reproduction is dependent upon the quality of the copy submitted.

In the unlikely event that the author did not send a complete manuscript
and there are missing pages, these will be noted. Also, if material had to be removed,
a note will indicate the deletion.

UMI U593418
Published by ProQuest LLC 2013. Copyright in the Dissertation held by the Author.
Microform Edition © ProQuest LLC.
All rights reserved. This work is protected against
unauthorized copying under Title 17, United States Code.

ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

I, Rebekka Andrea Vera Schwab, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

R. Schwab

ABSTRACT

The transcription factor B-MYB is involved in the regulation of proliferation, differentiation and apoptosis. Several lines of evidence indicate that aberrant expression of B-MYB might contribute to cancer. A link between B-MYB and neuroblastoma, a paediatric malignancy derived from the sympathoadrenal cell lineage, was proposed as B-MYB expression in primary neuroblastoma biopsies was significantly correlated with reduced patient survival. While attempting downregulation of B-MYB by RNAi techniques for therapeutic purposes, we found that the B-MYB protein is highly stable in neuroblastoma cell lines and increased B-MYB stability correlates with enhanced cell survival after UV-treatment. Increased B-MYB stability may be functionally relevant as cell survival is augmented upon ectopic B-MYB expression and decreased in the presence of dominant negative B-MYB. By comparing the B-MYB phosphorylation levels in a number of cancer cell lines, we detected that B-MYB phosphorylation is low in neuroblastoma cells and inhibition of B-MYB phosphorylation renders the protein more stable. We show that the phosphorylation deficient B-MYB mutant is functionally active in promoting cell survival after UV-irradiation and thus non-degradable B-MYB could promote survival of cells bearing genomic lesions that would normally be eliminated by cell death. Whereas no gross sequence abnormalities were identified, sequencing of the coding region of B-MYB from various neuroblastoma cell lines revealed two single nucleotide polymorphisms (SNPs). These do not contribute to enhanced B-MYB stability, but both polymorphic variants are hypomorphic and are significantly less frequent in a cohort of cancer patients, including neuroblastoma patients. This suggests that individuals with these B-MYB variants may have a lower incidence of cancer. Our results indicate that under certain circumstances, high doses of wild type B-MYB, potentially resulting from abnormal protein stabilisation, can facilitate transformation by promoting cell survival, but similar levels of the polymorphic variants may not be as detrimental.

ACKNOWLEDGEMENTS

I would like to express gratitude to my supervisor Arturo Sala for his excellent guidance and support throughout my PhD. I am grateful to Hugh Brady for critically evaluating my project. I would also like to thank all my colleagues in the Molecular Haematology and Cancer Biology unit for their friendship and helpful suggestions. I particularly thank Nipurna Jina and Anna Ruiz for enjoyable coffee and lunch breaks.

I am grateful to Giorgia Santilli who has given me a good introduction to the lab and I would like to thank Daisy Corvetta and Olesya Chayka for spicing up the Sala group.

I thank Jo Sinclair who provided expert assistance with flow cytometry and Liz Bland for advice with the heteroduplex analysis. I am grateful to Tim Cole for support with the statistical analysis.

I would like to thank the Neuroblastoma Society for financially supporting this work.

My special thanks go to my parents Heidi and Ruedi and my sister Ariane for their unconditional love, support and encouragement. I am particularly grateful to Christian Siebold who has the unique gift to make everything better.

This thesis is dedicated to my grandparents, Grosi Anna and Dätä Albert

CONTENTS

1 INTRODUCTION 15

1.1 TRANSCRIPTIONAL REGULATION 15
1.1.1 The eukaryotic promoter 15
1.1.2 The basal transcription machinery 18
1.1.2.1 Members of the PIC and their function 19
1.1.3 Transcription factors 21
1.1.3.1 Regulation of transcription factors 21
1.1.3.2 Cell type-specific expression of transcription factors 23
1.1.3.3 Transcription factors are modular proteins 23
1.1.3.3.1 The transactivation domain 23
1.1.3.3.2 The DNA binding domain 24
1.1.4 Transcriptional co-factors 26
1.1.4.1 The Mediator complex 27
1.1.4.2 Chromatin remodelling 27
1.1.5 Transcription factors and their role in cancer 29
1.1.5.1 Mechanisms involved in abnormal transcription factor function. 30
1.1.5.2 Aspects of transformation 32
1.1.5.2.1 Cell cycle progression is regulated by growth factors and inhibitors 32
1.1.5.2.2 Cell survival 36
1.1.5.2.3 Limitless replicative potential 38
1.1.5.2.4 Angiogenesis 40
1.1.5.2.5 Invasion and metastasis 41
1.2 B-MYB 44
1.2.1 The MYB family of transcription factors 44
1.2.2 Expression patterns of the vertebrate MYB family members 45
1.2.3 Evolutional development of the MYB family: important lessons from an inconspicuous organism 47
1.2.4 B-MYB structure 47
1.2.4.1 The DNA binding domain 48
1.2.4.2 The transactivation domain 49
1.2.4.3 The regulatory domain 49
1.2.5 Regulation of B-MYB 50
1.2.5.1 The B-MYB promoter and its transcriptional regulation 50
1.2.5.2 Post-translational modifications regulate B-MYB activity 54
1.2.5.2.1 B-MYB phosphorylation 54
1.2.5.2.2 B-MYB acetylation 57
1.2.5.2.3 B-MYB ubiquitination 57
1.2.5.3 Cooperation with co-factors 58
1.2.5.4 Interaction with regulatory proteins 60
1.2.6 Upstream pathways regulating B-MYB 63
1.2.7 B-MYB function 64
1.2.7.1 B-MYB regulates gene expression 64
1.2.7.2 Cell cycle control 71
1.2.7.3 Differentiation 74

1.2.7.4 Apoptosis 75
1.2.8 Is B-MYB a potential oncogene? *76*
1.3 NEUROBLASTOMA 80
1.3.1 Neural crest cells *81*
1.3.2 Disease staging and treatment strategies *82*
1.3.3 Genetic abnormalities occurring in neuroblastoma *86*
1.3.3.1 Chromosomal abnormalities 87
1.3.3.1.1 Ploidy 87
1.3.3.1.2 Amplification of 17q 87
1.3.3.1.3 Deletion of 1p 88
1.3.3.1.4 Deletion of 11q 89
1.3.3.1.5 Other chromosomal alterations 90
1.3.3.2 Altered gene expression 91
1.3.3.2.1 N-MYC amplification 92
1.3.3.2.2 Neurotrophin receptors 94
1.3.3.2.3 Other abnormal patterns of gene expression 96
1.4 THE CONNECTION BETWEEN B-MYB AND NEUROBLASTOMA 98
1.5 AIMS OF THE THESIS 100

2 MATERIALS AND METHODS 101

2.1 REAGENTS 101
2.2 BUFFERS AND SOLUTIONS 102
2.3 CELL BIOLOGY METHODS 103
2.3.1 Cell culture *103*
2.3.2 Retroviral infection *103*
2.3.3 Plasmid transfection *104*
2.3.4 siRNA oligonucleotide transfection *105*
2.3.5 Proliferation assay *106*
2.3.6 MTS assay *106*
2.3.7 Quantification of cell death *106*
2.3.7.1 Trypan blue exclusion 106
2.3.7.2 Single cell measurement of genomic DNA fragmentation 107
2.3.8 Immunofluorescence staining *107*
2.3.9 Cell treatments *108*
2.3.9.1 Drug treatments 108
2.3.9.2 Selection 108
2.3.9.3 UV-irradiation 108
2.4 PROTEIN BIOLOGY TECHNIQUES 109
2.4.1 Immunoblotting *109*
2.4.1.1 Sample preparation 109
2.4.1.2 Determination of protein concentration 109
2.4.1.3 Western blotting 109
2.4.2 Dual-luciferase reporter assay *110*
2.4.3 Expression and purification of GST-fusion proteins *111*
2.4.4 GST-Pin1 pull down *111*
2.5 MOLECULAR BIOLOGY TECHNIQUES 112
2.5.1 Plasmid preparation *112*
2.5.2 Preparation and transformation of chemically competent Escherichia coli *112*
2.5.3 shB-MYB construct cloning *112*

2.5.4 Generation of B-MYB variant constructs *113*
2.5.5 Isolation of genomic DNA *114*
2.5.6 Preparation of RNA and cDNA synthesis *114*
2.5.7 Quantitative PCR *115*
2.5.8 Sequencing of B-MYB *115*
2.5.9 Assessment of SNP status in neuroblastoma case-control samples by multiplex capillary heteroduplex analysis (MCHA) *117*
2.5.9.1 Origin of neuroblastoma patient and control samples 117
2.5.9.2 PCR 117
2.5.9.3 Heteroduplex formation 118
2.5.9.4 Capillary electrophoresis 118
2.5.9.5 Analysis of heteroduplex data 119
2.6 STATISTICAL ANALYSIS 119

3 RESULTS 120

3.1 ASSESSING THE THERAPEUTIC POTENTIAL OF TARGETING B-MYB BY SIRNA IN NEUROBLASTOMA 120
3.1.1 Background *120*
3.1.2 B-MYB is resistant to siRNA-mediated down-regulation in neuroblastoma cells *121*
3.1.3 Downregulation of B-MYB by shRNA *123*
3.1.4 Assessing B-MYB protein stability in neuroblastoma cell lines *126*
3.1.5 Discussion *128*
3.2 EVALUATING THE BIOLOGICAL CONSEQUENCES OF INCREASED B-MYB PROTEIN STABILITY 134
3.2.1 Background *134*
3.2.2 Increased B-MYB stability in UV-irradiated neuroblastoma cells *135*
3.2.3 Increased B-MYB stability after UV-irradiation is a combined effect of transcription induction and protein turnover *137*
3.2.4 Neuroblastoma cells are resistant to UV-induced apoptosis *139*
3.2.5 Effect of B-MYB overexpression in UV-sensitive Ewing sarcoma cells *141*
3.2.6 Use of a dominant-negative B-MYB construct to suppress B-MYB target gene expression in neuroblastoma cells *143*
3.2.7 Discussion *144*
3.3 MECHANISMS OF B-MYB STABILISATION IN NEUROBLASTOMA CELLS 148
3.3.1 Background *148*
3.3.2 Proteasomal degradation of B-MYB *149*
3.3.3 B-MYB phosphorylation *150*
3.3.4 Phosphorylation deficient B-MYB protein is more stable after UV-irradiation and confers protection from UV-induced apoptosis *153*
3.3.5 Discussion *155*
3.4 ANALYSIS OF B-MYB MUTATIONS IN NEUROBLASTOMA CELLS 161
3.4.1 Background *161*
3.4.2 B-MYB sequencing *161*
3.4.3 Single amino acids exchanges in the B-MYB protein do not alter protein stability *166*
3.4.4 Discussion *167*
3.5 FUNCTIONAL ANALYSIS OF B-MYB VARIANTS 170
3.5.1 Background *170*

3.5.2 B-MYB variants protect cells less efficiently from H_2O_2-induced cell death 171
3.5.3 Altered transactivation activity of B-MYB variants 174
3.5.4 The B-MYB variants localise exclusively to the nucleus 176
3.5.5 The different B-MYB forms display a similar overall phosphorylation status 177
3.5.6 Discussion 178
3.6 ASSESSMENT OF B-MYB VARIANT FREQUENCY IN NEUROBLASTOMA PATIENTS 184
3.6.1 Background 184
3.6.2 Setup of a large scale screening method for B-MYB and preliminary analysis of neuroblastoma samples 185
3.6.3 Assessing the frequency of two SNPs in the B-MYB coding region ... 192
3.6.4 Results from our collaborators 194
3.6.5 Discussion 195
4 CONCLUSION 202
5 REFERENCES 206

LIST OF FIGURES

FIGURE 1-1 CIS-ACTING ELEMENTS REGULATE TRANSCRIPTION 17
FIGURE 1-2 TRANSCRIPTION FACTORS CONTROL PIC FUNCTION 20
FIGURE 1-3 SCHEMATIC DOMAIN ORGANISATION OF THE B-MYB PROTEIN 48
FIGURE 1-4 TRANSCRIPTION OF B-MYB IS GOVERNED BY KEY PLAYERS OF THE CELL CYCLE 52
FIGURE 1-5 B-MYB IS SUBJECTED TO VARIOUS POST-TRANSLATIONAL MODIFICATIONS 56
FIGURE 1-6 SCHEMATIC MODEL ILLUSTRATING THE RELATIONSHIP BETWEEN B-MYB AND CO-FACTORS 59
FIGURE 1-7 OVERVIEW OF B-MYB REGULATORY PROTEINS 61
FIGURE 1-8 MIGRATION OF THE NEURAL CREST CELLS 81
FIGURE 3-1 NEUROBLASTOMA CELLS ARE RESISTANT TO SIRNA-MEDIATED DOWNREGULATION OF B-MYB 122
FIGURE 3-2 STABLE EXPRESSION OF SHB-MYB AFFECTS NEUROBLASTOMA CELL PROLIFERATION AND INDUCES APOPTOSIS 125
FIGURE 3-3 B-MYB PROTEIN DISPLAYS AN INCREASED HALF LIFE IN NEUROBLASTOMA CELL LINES 127
FIGURE 3-4 B-MYB IS RESISTANT TO PROTEASOMAL DEGRADATION INDUCED BY UV-IRRADIATION IN NEUROBLASTOMA CELLS 137
FIGURE 3-5 B-MYB MRNA IS INDUCED AFTER GENOTOXIC DAMAGE 138
FIGURE 3-6 NEUROBLASTOMA CELLS ARE RESISTANT TO UV-INDUCED APOPTOSIS 140
FIGURE 3-7 UV-IRRADIATION INDUCES B-MYB DEGRADATION IN THE ABSENCE OF APOPTOSIS 141
FIGURE 3-8 OVEREXPRESSION OF B-MYB TRANSIENTLY PROTECTS EWING SARCOMA CELLS FROM UV-INDUCED CELL DEATH 142
FIGURE 3-9 SUPPRESSING B-MYB TRANSACTIVATION ACTIVITY SLIGHTLY SENSITISES NEUROBLASTOMA CELLS TO UV-INDUCED CELL DEATH 144
FIGURE 3-10 B-MYB IS STABILISED BY THE PROTEASOME INHIBITOR MG-132 IN EWING SARCOMA BUT NOT IN NEUROBLASTOMA CELL LINES 150
FIGURE 3-11 B-MYB IS HYPOPHOSPHORYLATED IN NEUROBLASTOMA CELL LINES 152
FIGURE 3-12 A PHOSPHORYLATION-DEFICIENT B-MYB MUTANT IS RESISTANT TO UV-INDUCED DEGRADATION AND CONFERS PROTECTION FROM APOPTOSIS 154
FIGURE 3-13 STRATEGY FOR SEQUENCING HUMAN B-MYB AND IDENTIFICATION OF TWO POLYMORPHISMS IN NEUROBLASTOMA CELL LINES 163
FIGURE 3-14 THE IDENTIFIED SNPS ARE NON-SYNONYMOUS 164
FIGURE 3-15 B-MYB VARIANTS DO NOT AFFECT PROTEIN STABILITY 167
FIGURE 3-16 THE DIFFERENT FORMS OF B-MYB CONFER PROTECTION FROM H_2O_2-INDUCED CELL DEATH 172
FIGURE 3-17 THE B-MYB VARIANTS PROTECT CELLS LESS EFFICIENTLY FROM H_2O_2-INDUCED CELL DEATH 173
FIGURE 3-18 THE TRANSACTIVATION CAPACITY OF THE B-MYB VARIANTS IS REDUCED 175
FIGURE 3-19 B-MYB VARIANTS ARE PREDOMINANTLY LOCALISED TO THE NUCLEUS 177
FIGURE 3-20 THE B-MYB VARIANTS ARE SIMILARLY PHOSPHORYLATED 178

FIGURE 3-21 SCHEMATIC OVERVIEW OF DIFFERENT CLASSES OF SNPS 184
FIGURE 3-22 SCHEMATIC OVERVIEW OF THE PRINCIPLES OF HETERODUPLEX ANALYSIS 187
FIGURE 3-23 STRATEGY FOR ANALYSING CDNA SAMPLES FOR B-MYB SEQUENCE VARIATIONS BY MCHA 188
FIGURE 3-24 ASSESSING THE SENSITIVITY OF MCHA 189
FIGURE 3-25 STRATEGY FOR ANALYSING B-MYB SEQUENCE VARIATIONS FROM GENOMIC DNA SAMPLES BY MCHA 191

LIST OF TABLES

TABLE 1-1 INSS 83
TABLE 1-2 CHARACTERISTICS OF NEUROBLASTOMA RISK GROUPS 85
TABLE 2-1 BUFFERS AND SOLUTIONS 102
TABLE 2-2 CELL LINES 103
TABLE 2-3 PLASMIDS USED FOR RETROVIRAL INFECTIONS 104
TABLE 2-4 PLASMIDS USED FOR TRANSFECTIONS 105
TABLE 2-5 ANTIBODIES USED FOR IMMUNOFLUORESCENCE STAINING 108
TABLE 2-6 ANTIBODIES USED FOR WESTERN BLOTTING ANALYSIS 110
TABLE 3-1 PERCENTAGE OF ACTIVATED CASPASE-3 POSITIVE CELLS 174
TABLE 3-2 ASSOCIATION BETWEEN THE G427 B-MYB POLYMORPHISMS AND INCIDENCE OF NEUROBLASTOMA 193
TABLE 3-3 ASSOCIATION BETWEEN THE M624 B-MYB POLYMORPHISMS AND INCIDENCE OF NEUROBLASTOMA 193
TABLE 3-4 ASSOCIATION BETWEEN ANY B-MYB POLYMORPHISM AND INCIDENCE OF NEUROBLASTOMA 193
TABLE 3-5 ASSOCIATION BETWEEN THE G427 B-MYB POLYMORPHISMS AND INCIDENCE OF CML 195
TABLE 3-6 ASSOCIATION BETWEEN THE M624 B-MYB POLYMORPHISMS AND INCIDENCE OF CML 195
TABLE 3-7 ASSOCIATION BETWEEN ANY B-MYB POLYMORPHISM AND INCIDENCE OF CML 195

ABBREVIATIONS

APS	ammonium persulfate
BDNF	brain-derived neurotrophic factor
BRE	TFIIB-responsive element
cAMP	cyclic adenosine monophosphate
Cdk	cyclin dependent kinase
cDNA	complementary DNA
CHD	chromodomain helicase DNA binding domain
ChIP	chromatin immunoprecipitation
CHX	cycloheximide
CML	chronic myeloid leukaemia
CR	conserved region
cSNP	SNP occurring in a coding region
DAPI	4,6-diamindino-2-phenylindole-2
DBD	DNA binding domain
DM	double-minute chromosome body
D-MEM	Dulbecco's modified Eagle's medium
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
ECM	extracellular matrix
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
EMSA	electrophoretic mobility shift assay
ES cell	embryonic stem cell
FACS	fluorescence activated cell sorter
FADD	Fas-associated death domain
FBS	foetal bovine serum
FGF	fibroblast growth factor
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GTF	general transcription factor
HAT	histone acetyltransferase
HDAC	histone deacetylase

HLH	helix-loop-helix
HRP	horse-radish-peroxidase
HSE	heat shock element
HSP	heat shock protein
HSR	homogeneously staining region
hTERT	human telomerase reverse transcriptase
HTH	helix-turn-helix
ICM	inner cell mass
IGF	insulin-like growth factor
IGFBP	insulin-like growth factor-binding protein
IL	interleukin
Inr	initiation region
INSS	International Neuroblastoma Staging System
IP	immunoprecipitation
LB	Luria-Bertani
LCR	locus control region
MBS	MYB binding site
MCHA	multiplex capillary heteroduplex analysis
MEF	mouse embryonic fibroblast
MIBG	meta-iodobenzyl-guanidine
MMP	matrix metalloproteinase
mRNA	messenger RNA
NCBI	National Centre for Biotechnology Information
NGF	nerve growth factor
NLS	nuclear localisation signal
NT	neurotrophin
p75NTR	p75 neurotrophin receptor
PCR	polymerase chain reaction
PI3K	phosphatidylinositol 3-kinase
PIC	preinitiation complex
POLA	DNA polymerase α
RA	retinoic acid
RNA	ribonucleic acid
RNAi	RNA interference

RNAP	RNA polymerase
ROS	reactive oxygen species
rRNA	ribosomal RNA
rSNP	SNP occurring in a non-coding region
RT-PCR	real time PCR
shRNA	short hairpin RNA
siRNA	small interfering RNA
SMC	smooth muscle cell
SNP	single nucleotide polymorphism
SP-A	surfactant protein-A
TAF	TBP associated factor
TBP	TATA-binding protein
TEMED	N,N,N',N'-tetramethylethylenediamine
TOP2A	DNA topoisomerase IIα
tRNA	transfer RNA
UPE	upstream promoter element
UV	ultraviolet
VEGF	Vascular endothelial growth factor

1 INTRODUCTION

1.1 Transcriptional regulation

The generation of a protein from its blueprint, the gene, is a multistep process, including transcription of the gene in to messenger RNA (mRNA) and subsequent translation, which involves protein assembly from amino acids at ribosomes in the cytoplasm. While some proteins are synthesised at a constant rate or in a specific pattern during, for example, certain stages of development or during particular phases of the cell cycle, others are inducible. Especially during development, but also during adult life, a tight control of gene expression is required. Abnormal gene expression has been linked to a number of diseases such as cancer. Most cancers arise from aberrant expression of genes involved in growth control, cell death and differentiation. These genes are normally dubbed proto-oncogenes or tumour suppressor genes. Thus, tightly controlled gene expression in healthy organisms ensures the presence of a specific set of proteins at a certain moment in time, guaranteeing appropriate differentiation and rapid response to environmental influences. To warrant tight regulation of gene expression, control is maintained at different levels. These include control of chromatin packaging, transcriptional regulation, stability and processing of the pre-mRNA and regulation of protein synthesis.

1.1.1 The eukaryotic promoter

The process of gene transcription in eukaryotes is subdivided into initiation of transcription, followed by transcription elongation and termination. Transcription initiation by RNA polymerase II (RNAP II) occurs at specific sites called promoters, which are control regions in the immediate vicinity of a transcription start site (Mitchell and Tjian, 1989). Typical features of a eukaryotic promoter are core elements including an AT-rich sequence called the TATA box, a TFIIB-responsive element (BRE), and an initiation region (Inr) (Figure 1-1) that encompasses the start consensus nucleotide triplet ATG. It is by no means obligatory that a promoter must contain all three elements. In fact, most eukaryotic

protein encoding genes contain TATA-directed promoters whereas Inr-directed promoters are less frequent and promoters with none of these elements often have multiple transcription start sites (Lee and Young, 2000). The core elements comprise approximately 100 base pairs and are the site of assembly for the transcription pre-initiation complex (PIC), which consists of the RNAP II and several general transcription factors (GTFs). The core elements of a promoter in combination with the PIC allow basal transcription of a gene (Martinez, 2002; Lee and Young, 2000).

Apart from core elements, a typical eukaryotic gene is regulated by additional cis-regulatory elements, which are located both upstream and downstream of the transcription start site. Each gene in an animal cell has a particular combination of positive and negative cis-regulatory elements that are unique in number, type and location. They are the binding sites for transcriptional regulators, commonly called transcription factors from where they regulate transcription (Mitchell and Tjian, 1989).

Among these cis-regulatory sequences are upstream promoter elements (UPEs), usually located immediately upstream of the promoter region (Figure 1-1) (Lee and Young, 2000). UPEs of many genes contain the CCAAT box that provides a binding site for a variety of different transcription factors and/or a GC-rich sequence that is bound by the ubiquitous and constitutively expressed transcription factor Sp1 (Barberis and Petrascheck, 2003).

In addition, some genes contain regulatory sequences, which bind transcription factors that become active only in response to particular stimuli. This signal-dependent transcriptional regulation involves responses to the presence of signalling molecules such as hormones and growth factors or to stress conditions such as heat shock (Mitchell and Tjian, 1989). Genes induced by heat shock commonly contain a so called heat shock element (HSE) and introduction of this element in the promoter of the thymidine kinase gene rendered it inducible by elevated temperatures (Pelham and Bienz, 1982). A number of other elements that confer responses to particular stimuli, such as cyclic adenosine monophosphate (cAMP) (Horiuchi et al., 1993) or glucocorticoid hormone (Fan et al., 1992), have been identified.

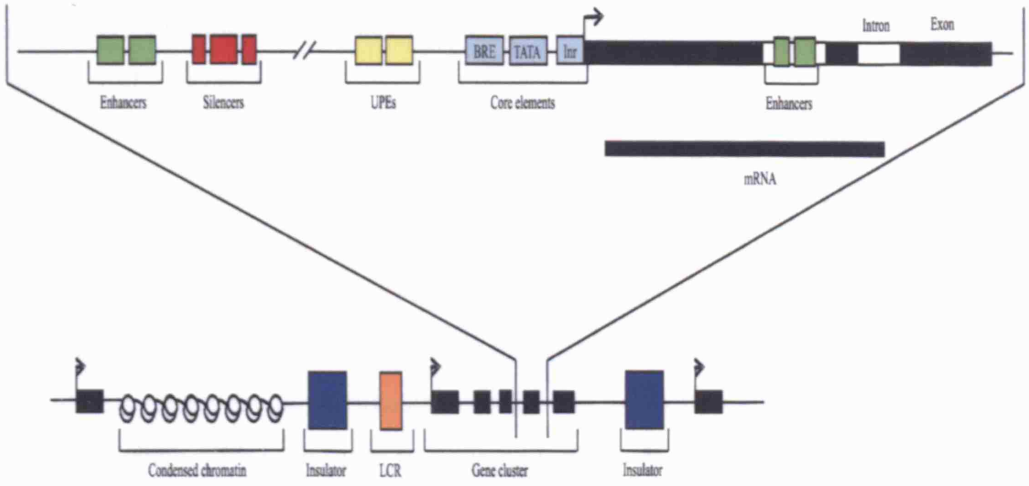


Figure 1-1 Cis-acting elements regulate transcription

A typical eukaryotic promoter consists of various regulatory sequences. The core promoter is where the PIC assembles and may encompass the TATA box, the TFIIB-responsive element (BRE) and the initiation region (Inr) (light blue boxes). Further regulatory sequences include upstream promoter elements (UPEs) (yellow boxes), enhancers (green boxes) and silencers (red boxes), which act independently of their location and orientation. The locus control region (LCR) (orange box) enhances expression of a cluster of genes and insulators (dark blue boxes) shield genes from neighbouring regulatory elements and chromatin condensation. Figure adapted according to (Lemon and Tjian, 2000; Bell et al., 2001).

While all elements described so far are located close to the transcription start site, transcription of eukaryotic genes can also be regulated from remote sites, known as enhancers (Figure 1-1) (Lee and Young, 2000). Enhancers can function when located at distances of several kilo bases away from the start site, upstream or downstream of a gene and independently of their orientation. Although these regulatory elements cannot drive transcription themselves, they can greatly augment the activity of promoters (Barberis and Petrascheck, 2003). Silencers (Figure 1-1) are also able to act in an orientation- and position-independent manner, but instead of activating transcription of a particular gene, they repress promoter activity (Lee and Young, 2000). The silencing transcription factor REST for

example, has been shown to bind to a silencer DNA element termed RE1 that controls expression of the type II voltage-dependent sodium channel gene. REST is only present in non-neuronal cells were it binds to RE1 and blocks gene expression (Chong et al., 1995).

There are further forms of control of gene expression. Clusters of genes have been characterised that respond to locus control regions (LCR) consisting of a number of DNAse I hypersensitivity sites (Figure 1-1). LCRs are defined by their ability to enhance the expression of linked genes in a tissue-specific manner and they execute their effects over very large distances. The LCR was first identified in the human β-globin locus where it is thought to open and maintain a permissive chromatin conformation in erythroid cells. The human β-globin locus consists of five functional genes and although each globin gene within the cluster is individually regulated, the LCR is necessary for the expression of all globin genes (Li et al., 2002). Conversely, some genes are flanked by insulator elements (Figure 1-1) that provide a barrier against influences from the surrounding DNA. The boundary function of some insulators may be modified in a regulated fashion by proteins that either bind adjacent regulatory sites or by insulator bound proteins (Bell et al., 2001).

1.1.2 The basal transcription machinery

In total, three distinct RNAPs were purified due to differences in their nuclear location, chromatographic behaviour, salt requirements, subunit composition, sensitivity to the toxin α-amanitin and most importantly the class of RNA they synthesise (Archambault and Friesen, 1993). In contrast to RNAPs I and III, which are responsible for transcription of genes encoding ribosomal RNA (rRNA) and transfer RNAs (tRNAs), respectively, RNAP II transcribes protein encoding genes and is in charge of the entire mRNA synthesised of a cell. RNAP II consists of several subunits, which are highly conserved among eukaryotes (Lee and Young, 2000).

1.1.2.1 Members of the PIC and their function

Unlike the bacterial RNA polymerase, purified RNAP II cannot specifically initiate transcription solely with naked DNA. Notably, constitutively expressed GTFs are necessary for this process and they interact directly or indirectly with the RNAP II (Lee and Young, 2000). The following proteins are referred to as GTFs: TFIIA, TFIIB, TFIID, TFIIE, TFIIF and TFIIH (Figure 1-2). All human GTFs, with the exception of TFIIB, are complexes formed by stable association of different proteins (Martinez, 2002). Similar to RNAP II, GTFs are remarkably conserved among eukaryotes and they are involved in processes such as positioning the RNAP II at gene promoters, opening the two DNA strands and enabling initiation and elongation of transcription (Lee and Young, 2000).

Transcription is initiated with the recruitment of the PIC to the promoter whereby PIC-assembly occurs in a stepwise manner *in vitro*: Firstly, the TFIID protein complex consisting of the TATA box binding protein (TBP) and several TBP associated factors (TAFs) are assembled on the TATA box (Woychik and Hampsey, 2002). TBP is required for initiation by all three RNA polymerases and binding of TBP results in structural alteration of the underlying DNA. This change in DNA structure provides the appropriate topology for assembly of further GTFs (Hampsey and Reinberg, 2001). Following TFIID binding, TFIIA and TFIIB associate with the growing complex and stabilise the binding of TFIID to the DNA. Like TBP, TFIIB is a sequence-specific GTF that binds to the promoter element BRE and it is thought that the interaction of TFIIB with BRE is important in determining the polarity of the PIC (Woychik and Hampsey, 2002). Assembly of the PIC continues with TFIIF binding to RNAP II and escorting it to the complex. TFIIF was further implicated in preventing RNAP II from non-specific DNA binding, stabilisation of the PIC, start site recognition as well as stimulation of transcription elongation (Lee and Young, 2000). Finally, TFIIE and TFIIH are sequentially recruited to the complex, whereas TFIIE is important for the recruitment and subsequent regulation of TFIIH activities (Woychik and Hampsey, 2002). Moreover, both TFIIE and TFIIH are required for ATP-dependent formation of the open promoter complex (Woychik and Hampsey, 2002). TFIIH is the largest and most complex member of the GTFs and possesses helicase properties that

enable it to unwind DNA downstream of the initiation site. Importantly, the TFIIH complex also contains a kinase subunit that phosphorylates the C-terminal domain of RNAP II (Woychik and Hampsey, 2002).

Formation of a stable initiation complex is followed by promoter clearance and elongation to effectively produce an mRNA transcript. Phosphorylation of the RNAP II C-terminal domain provides the signal for the switch from initiation to elongation (Martinez, 2002). During mRNA synthesis, RNAP II together with TFIIF and additional elongating factors extend the transcript downstream of the promoter. As transcription elongation proceeds, RNA capping, splicing and ultimately polyadenylation are carried out by factors that have been found associated with the phosphorylated RNAP II (Lee and Young, 2000; Martinez, 2002).

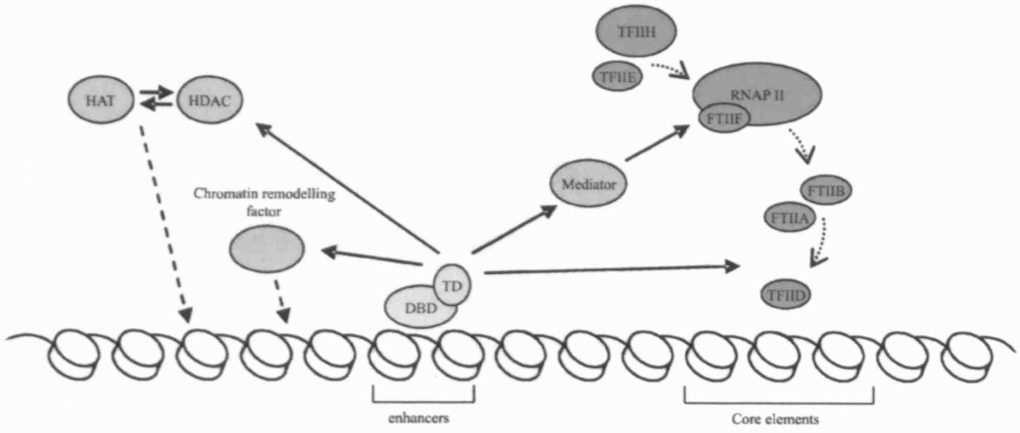


Figure 1-2 Transcription factors control PIC function

Transcription factors enhance transcription by binding to DNA and interacting with a vast array of different proteins. Transcription factors (yellow) composed of DNA binding (DBD) and transactivation domains (TD) interact directly or indirectly via co-factors (blue) with members of the PIC (green). These interactions stimulate PIC assembly or activation. Chromatin modifying, such as histone acetyltransferases (HATs) or histone deacetylases (HDAC), and chromatin remodelling factors are recruited to a promoter through their interaction with transcription factors. The action of these factors renders the tightly packed chromatin structure accessible for further components of the transcription machinery.

Uninterrupted arrows represent interactions between transcription factors with target proteins, dotted arrows indicate assembly of the PIC and dashed arrows symbolise modification of the chromatin structure.

1.1.3 Transcription factors

Basal transcription can be modulated through the action of transcription factors (Figure 1-2). Unlike GTFs, which are common to all RNAP II transcribed genes, transcription factors are gene-specific. They interact with specific DNA elements to elevate or repress the basal rate of transcription. Generally, transcription factors regulate transcription by recruiting chromatin-modifying factors that allow access to a gene, by guiding the transcriptional machinery to promoters or by increasing the elongation rate of RNAP II (Lee and Young, 2000). Promoters of higher eukaryotes are composed of binding sites for multiple transcription factors, which in turn cooperate with one another to activate transcription synergistically (Mitchell and Tjian, 1989).

1.1.3.1 Regulation of transcription factors

The balance between transcription factors that activate or repress determines the rate of transcription and, extracellular signals or cell type-specific programs may alter this balance. While the role of transcription factors is to regulate gene expression, they are tightly regulated proteins themselves. Many transcription factors are controlled at the level of synthesis, such as the homeodomain-containing transcription factors involved in development of multicellular organisms (Boncinelli, 1997). On the other hand, some transcription factors are responsible for an immediate response to certain stimuli. Such transcription factors usually persist in the cell in an inhibited form and become activated upon a specific stimulus. Several different mechanisms of pre-existing transcription factor activation have been identified: Ligand binding can release transcription factors bound to an inhibitor, thus allowing them to activate transcription. Steroid receptors belong to the family of nuclear receptors and steroid binding causes dissociation of the receptor from an inactive complex containing Hsp90. This leads to receptor

dimerization and binding to hormone response elements (Danielian et al., 1992). On the other hand, some transcription factors such as the NF-κB family members are kept in an inactive form in the cytoplasm through inhibitor binding. NF-κB consists of closely related protein dimers that bind a common DNA sequence motif. These dimers are retained in the cytoplasm through binding to their inhibitor IκB. Stimulation of this pathway results in phosphorylation and subsequent ubiquitin-dependent degradation of IκB. This in turn allows the NF-κB dimers to translocate to the nucleus and activate transcription (Karin et al., 2002). Moreover, post-translational modifications are important means to directly regulate the activity of transcription factors. The transcription complex AP-1 consisting of c-Jun and c-Fos family members is required for cell proliferation. AP-1 is induced in response to a wide variety of extracellular signals and one example is platelet-derived growth factor (PDGF), which regulates the expression of c-Fos. PDGF also initiates phosphorylation of the transactivation domain of c-Fos through the extracellular signal-regulated kinase (ERK) and phosphorylation of c-Fos increases its transactivating potential (Monje et al., 2003). Another mode of transcription factor regulation is exemplified by C/EPBβ, which is a member of the CCAAT/enhancer binding protein family of transcription factors that control expression of genes involved in proliferation and differentiation. C/EBPβ is activated through Ras-mediated MAPK signalling and activation of this pathway ultimately leads to a conformational change in C/EBPβ. Structural alteration of C/EBPβ induces dissociation from its repressor and subsequent interaction with transcriptionally active complexes (Mo et al., 2004). Alternatively, competition for dimerization partners can determine transcriptional regulation of a target gene. A well studied example is the MYC/MAX/MAD network of transcription factors that regulate growth, differentiation and apoptosis. MAX forms heterodimers with MYC, which stimulate transcription. Contrarily, MAX is also able to dimerise with MAD and although these complexes bind to the same regulatory element as the MAX/MYC heterodimers, formation of MAX/MAD dimers results in repression of transcription. Whereas MYC and MAD genes and proteins are highly regulated, MAX expression is constitutive. Thus, the ratio of MYC and MAD in a cell decides, which complexes will preferentially form with MAX and thus result in transcriptional activation or repression (Ayer et al., 1993).

1.1.3.2 Cell type-specific expression of transcription factors

In contrast to transcription factors, which are widely expressed in different cell types, certain transcription factors are solely produced in specific tissues. Cell type-specific transcription factors bind to regulatory elements of one or several given genes, thus participating in differentiation or executing specialised function. MyoD is one of the first tissue-specific transcription factors identified. MyoD is synthesised in skeletal muscle cells and regulates genes, which only occur in these cells. The critical role of this transcription factor in muscle cells was demonstrated by overexpressing MyoD in undifferentiated fibroblasts, which converted them into postmitotic myoblasts (Choi et al., 1990). Another example of tissue-specific transcription factors is the MYB family, which comprises three different members. Interestingly, two members of this family of transcription factors, c-MYB and A-MYB are expressed in a tissue-specific manner, whereas expression of the third member of the family, B-MYB is ubiquitous (Oh and Reddy, 1999).

1.1.3.3 Transcription factors are modular proteins

Eukaryotic transcription factors are modular proteins that are typically composed of a sequence-specific DNA binding domain (DBD) and an activation domain, which is also referred to as a transactivation domain. While DNA binding alone is not sufficient for activating transcription, anchoring the transcription factor to DNA through the DBD orients the transactivation domain and thus allows it to induce transcription.

1.1.3.3.1 The transactivation domain

Transcription factors interact through their transactivation domain directly or indirectly with members of the PIC to stimulate or repress transcription. Such interactions have been revealed for subunits of the TFIID complex (TBP and TAFs) and some other GTFs (Barberis and Petrascheck, 2003). Transactivation domains were identified to stimulate transcription when linked to the DBD of completely unrelated transcription factors (Mitchell and Tjian, 1989). Transactivation domains consist of as few as 30 to 100 amino acids and transcription factors often contain more than one. Several structural motifs have been identified that confer

transactivation and according to a predominance of particular amino acid residues, transactivation domains have been classified as acidic, glutamine- or proline-rich (Mitchell and Tjian, 1989). A prominent example for a transcription factor with an acidic transactivation domain is p53, a tumour suppressor, which is mutated in a large variety of human tumours (Fields and Jang, 1990). On the other hand, the human GC box-binding transcription factor Sp1 contains two glutamine-rich transactivation domains. Interactions between these domains with the TBP-associated factor dTAFII110 is critical for transcriptional activation by Sp1 (Gill et al., 1994). On the contrary, Smad4, which is a mediator of cytokine signalling responses such as TGF-β contains a proline-rich transactivation domain. Interaction between the transactivation domain of Smad4 with CBP/p300 was found to be essential for efficient transcriptional activation (de Caestecker et al., 2000).

1.1.3.3.2 The DNA binding domain

In order for transcription factors to activate or repress transcription they need to bind to DNA sequences via their DBDs and the actual DNA binding involves protein subregions consisting of 60 to 100 amino acids. Transcription factors employ different structural motifs to form the protein-DNA interface. The formation of a specific protein-DNA complex usually involves conformational changes in both the protein and the DNA. Many transcription factors exist in dimers and bind DNA only in this form. On the basis of common evolutionary conserved motifs in the DBD, most mammalian transcription factors have been classified into families. The best studied families include transcription factors with a zinc finger motif, a leucine zipper motif, a helix-loop-helix motif (HLH) or a homeodomain (Mitchell and Tjian, 1989; Patikoglou and Burley, 1997).

The family of transcription factors with zinc finger motifs is subdivided into various classes (Pabo and Sauer, 1992). One of the most important zinc finger motifs consists of two cysteines and two histidines, which bind a zinc atom. The mammalian transcription factor Sp1 contains three such tandem zinc fingers at its carboxy-terminus, which are sufficient to bind DNA (Mitchell and Tjian, 1989). Another frequent type of zinc finger is exemplified by the DBD of steroid hormone receptors. When these receptors are activated by their cognate ligands they become

transcription factors themselves and bind to DNA via their zinc finger motif. These so called multicysteine zinc fingers are motifs containing four cysteines binding to a zinc atom (Mitchell and Tjian, 1989).
Transcription factors with a zinc finger motif have been implicated in several malignant diseases. The tumour suppressor gene WT1 is mutated in 10-15% of Wilms tumours and in 15% of acute myeloid leukaemias where it is associated with poor response to chemotherapy (Ladomery and Dellaire, 2002).

Many transcription factors contain a basic DBD, but this domain only binds to DNA when the transcription factor occurs as a dimer. Therefore, such transcription factors additionally require a dimerization region. Dimerization is achieved either through a leucine zipper or a HLH motif. A leucine zipper motif is a short region consisting of an α-helix in which the leucines occur on one side of the helix. This structure can bind to the same motif of another transcription factor, thus allowing the formation of a dimer. The leucine zipper motif is found in a wide variety of transcription factors from fungi to plants and animals (Pabo and Sauer, 1992) and underlies the interactions between the proto-oncogene products c-Jun and c-Fos. Thus, the leucine zipper allows formation of the heterodimeric transcription factor AP-1, which is implicated in cellular growth and carcinogenesis (van Dam and Castellazzi, 2001). Alternatively, dimerization may be achieved by the HLH, whereby each amphipathic helix consists of hydrophobic residues on one side and charged residues on the other side connected by a loop of variable length (Pabo and Sauer, 1992). Transcription factors, which are members of the HLH family are involved in a wide array of developmental processes and members of the MYC/MAX/MAD network are presumably the best known examples of transcription factors with HLH motifs (Hurlin et al., 1996). Another example is the HLH HASH-1 protein whose expression correlates with immature neuroblastoma cells and is downregulated upon neuroblastoma cell differentiation (Axelson, 2004).

The helix-turn-helix (HTH) motif is the most common motif found in DNA binding proteins, mainly being present in prokaryotic repressors. It contains about 20 amino acids that form two α-helices with a short turn in between. This motif is also found in eukaryotes with some modifications though. The eukaryotic HTH motif is called homeodomain, encompasses about 60 amino acids and was first characterised in

several transcription factors involved in *Drosophila* embryogenesis (Mitchell and Tjian, 1989). The homeodomain recognises a DNA sequence named homeobox, which is present in a large number of genes. The homeodomain is folded into three α-helices with the second and third helices resembling the HTH motif. In contrast to the prokaryotic HTH, the homeodomain binds to DNA as a monomer (Mitchell and Tjian, 1989). Prominent members of the homeodomain family are the Hox genes, which are organised in four clusters in the human genome. These genes control the formation of the various regions along the body and therefore various congenital malformations are associated with defective Hox gene expression. Apart from development defects, Hox genes have also found to be involved in different cancers such as acute myeloid leukaemia in humans where a translocation involving HOXA9 is proposed to promote leukaemogenesis (Boncinelli, 1997).

Several other important transcription factors do not fall in any of the classes mentioned above. An example is the human tumour suppressor protein p53 that contains two loops coordinated by a zinc atom and a loop-sheet-helix motif, which constitutes its DBD (Cho et al., 1994). On the other hand, the proto-oncogenes of the vertebrate MYB family all contain a DBD, which is characterised by periodically arranged tryptophans (Oh and Reddy, 1999).

1.1.4 Transcriptional co-factors

Although transcription factors are able to change basal transcription levels *in vivo*, they are often unable to do so *in vitro*, and this suggests, that transcription factors may require the function of a variety of so-called co-factors. Co-factors play essential roles in mediating or facilitating the effects of transcription factors on the PIC, either via direct physical interactions with members of the PIC (Martinez, 2002) or indirectly through modification of the chromatin structure (Narlikar et al., 2002). Therefore, co-factors do not bind to DNA in a sequence-specific manner but are generally recruited to specific promoters via interactions with either transcription factors and/or components of the basal transcription machinery.

1.1.4.1 The Mediator complex

Co-factors can exert a global function and are likely to be recruited to most genes. The most universal co-factor is the Mediator complex (Figure 1-2), which transduces regulatory signals between transcription factors and the PIC (Woychik and Hampsey, 2002). Mediator is an evolutionarily conserved multiprotein complex that is ubiquitously expressed in eukaryotes, with different subunit compositions depending on the promoter, and plays important roles in the activation or repression of RNAP II (Lee and Young, 2000). Therefore, the Mediator complex transduces both positive and negative transcriptional signals through its adaptor function from transcription factors to the PIC. The Mediator complex was also found to reversibly associate with the unphosphorylated C-terminal domain of RNAP II, thus stimulating phosphorylation of RNAP II by the TFIIH-associated kinase (Conaway et al., 2005).

1.1.4.2 Chromatin remodelling

In contrast to an adaptor function, some co-factors can display enzymatic activities such as the ability to remodel or modify chromatin. This is particularly crucial, as DNA is packed into higher order structure in eukaryotic cells. The first order of DNA condensation is achieved by winding DNA around nucleosomes and this is referred to as chromatin. The nucleosome core is composed of two sets of four highly folded and much conserved proteins, H2H, H2B, H3, H4 and of 146 base pairs of DNA wound around the octamer. The second order of condensation consists of long arrays of nucleosomes, which are further folded and compacted through the binding of the linker histone H1 into chromatin fibres (Lee and Young, 2000). As a consequence, accessibility to DNA is significantly reduced and therefore transcription of a gene first requires remodelling of the chromatin to allow entry of the PIC. Some transcription factors, such as the transcription factor GATA-4, are able to bind to tightly condensed chromatin. Such transcription factors are often referred to as pioneer factors and their binding to compact chromatin opens up a local region (Cirillo et al., 2002). Other transcription factors use the opportunity of opened chromatin to access enhancer or promoter elements and further modify

and remodel chromatin by recruiting and targeting chromatin modifying and remodelling complexes.

Two major classes of co-factor complexes regulate accessibility of the DNA whereby one acts through covalent and the other through non-covalent modifications. Energy is used to modify the chromatin structure in a non-covalent manner and this is performed by so called chromatin remodelling factors (Figure 1-2), such as the Swi/Snf complex (Lee and Young, 2000). The best described mechanism involves sliding of the DNA with respect to the nucleosom, thereby rendering DNA sequences exposed or hidden. Short range sliding does not increase the amount of exposed DNA, it only changes its location (Narlikar et al., 2002).

The other class of factors that regulate DNA accessibility are proteins that covalently modify nucleosomes by adding or removing different chemical groups at the histone amino termini. The best characterised of these co-factors are those that cause either hyperacetylation or hypoacetylation of lysines in the amino-terminal tails of the core histone (Narlikar et al., 2002). These co-factors are dubbed histone acetyltransferases (HATs) and histone deacetylases (HDAC), respectively (Figure 1-2) and they are commonly associated with multiprotein complexes (Lee and Young, 2000). Examples for HAT complexes are TFIID with the active HAT subunit TAFII250 or the SAGA complex containing Gcn5 with HAT activity (Lee and Young, 2000). p300 and CBP are two highly related proteins that are bound by a variety of transcription factors. Importantly, these two transcriptional co-activators also have HAT activity (Lee and Young, 2000). In contrary, two prominent human HDACs are Sin3 and NuRD, which can be directly recruited by transcription factors or in a more indirect manner via co-repressors such as N-CoR and SMRT (Lee and Young, 2000).
Some transcription factors can associate with chromatin modifying HAT or HDAC complexes thereby recruiting their activities to specific locations. Recruitment of chromatin modifying complexes to promoter regions creates specific patterns of hyper- or hypoacetylation. Hyperacetylation has been linked to transcriptionally active states of chromatin and it is thought to induce a more open nucleosome conformation (Narlikar et al., 2002).

Accumulating evidence indicates that other modifications such as methylation, ubiquitination and phosphorylation are able to influence chromatin condensation as well (Berger, 2001). For example, phosphorylation of histones H1 and H3 is important in chromosome condensation during mitosis (Lee and Young, 2000), methylation of distinct lysines in histones H3 and H4 has been associated with gene silencing (Fuks, 2005) and reversible ubiquitination of histones H2A, H2B and H3 has been linked with DNA transcription (Lee and Young, 2000).

In summary, gene transcription is highly regulated by transcription factors, which are in turn assisted by a plethora of proteins involved in fine-tuning gene expression. Abnormal transcription factor regulation may affect the control of a large array of target genes and this can lead to disease including cancer. The connection between aberrantly regulated transcription factors and cancer is discussed in the next sections.

1.1.5 Transcription factors and their role in cancer

Generally, cancers originate from a single somatic cell and its resulting progenitor cells. While normal cells are under tight control of various mechanisms that prevent them from becoming malignant, transformation often results from aberrant activation of specific genes. These genes are known as proto-oncogenes, whose normal cellular function involves regulation of cell growth, cell survival and/or differentiation. Activated proto-oncogenes acquire a dominant gain of function and are commonly referred to as oncogenes (Ponder, 2001; Nebert, 2002). The counterpart of an oncogene is a tumour suppressor gene. Classical tumour suppressor genes are limiting for cancer development. However, the loss of many other genes that are not considered as classical tumour suppressor genes accelerates the acquisition of further genetic defects and these genes are generally involved in processes such as DNA repair or genome integrity (Ponder, 2001; Nebert, 2002). Transformation is a multi-step process whereby cells gradually acquire genetic alterations that drive the progressive transformation of normal human cells into highly malignant derivatives. Such genetic alterations allow circumventing several regulatory mechanisms that govern cell homeostasis (Ponder, 2001). Activation of

proto-oncogenes or inhibition of tumour suppressor genes may be achieved by abnormal protein expression/repression. This, in turn, might result in deregulated control of cellular proliferation, which initially leads to a highly proliferating cell population. This provides the base to further accumulate an array of genetic changes, until a cell population evolves which is freed from the normal constraints that are imposed on cells of a multicellular organism (Nebert, 2002).

Cancer occurs as a consequence of random mutations and, while more than one event is required for carcinogenesis, the number of mutational events depends on the cell type and the proliferation constraints that underlay the regulation of a particular tissue (Knudson, 2001). Furthermore, the function of a particular protein depends on the cellular background where it is expressed, as overexpression in one tissue may enhance but in another inhibit growth or even induce apoptosis (Weinstein, 2002).

During development, cells either differentiate along tissue-specific pathways or remain in the quiescent state from which they may re-emerge upon appropriate mitogenic stimulation. Some cancers occur almost exclusively in children and they often originate from tissues whose cells would have ceased to proliferate and undergone differentiation in an adult. In contrast to most adult tissues, cells from embryonic and postnatal tissues grow and divide rapidly and, therefore, it is generally believed that only a low number of mutations is required for the origin of paediatric cancers (Knudson, 2001).

1.1.5.1 Mechanisms involved in abnormal transcription factor function

Activation of proto-oncogenes or inhibition of tumour suppressor genes can manifest in different ways ranging from minimal DNA alterations, such as point mutations, to changes involving whole chromosomes. DNA from proliferating cells is particularly prone to mutations because the whole genome is replicated during every cell cycle. Alternatively, DNA damaging agents such as chemicals or radiation can provoke changes in the DNA sequence. Repair mechanisms ensure the integrity of the genome but if DNA alterations escape the repair machinery,

permanent mutations arise (Nebert, 2002). While germ line mutations are found in every cell descended from the zygote, somatic mutations manifest only in specific tissues. Consequently, somatic mutations are involved in the development of sporadic, whereas germ line mutations are responsible for inherited cancers (Knudson, 2001).

While mutational events are the basis for tumourigenesis, transcription factors do not necessarily need to be mutated for oncogenic activation. It may be sufficient when proteins that regulate transcription factors are mutated, thus conferring oncogenic properties on transcription factors. Nevertheless, several examples exist, where transcription factors were found to be mutated and therefore facilitate the development of cancer. Notably, mutations in the tumour suppressor p53 occur in more than 50% of spontaneous tumours and p53 was found to be mutated in the germ line of patients with Li-Fraumeni syndrome (Iwakuma et al., 2005). A majority of p53 alterations consist of missense mutations in the DBD that disrupt the ability of p53 to bind to DNA and activate transcription (Knudson, 2000). Another example is WT1, one of the best characterised transcription factors that has been shown to play a role in Wilms tumour. An array of genetic alterations involving missense, deletion or truncation mutations contribute to the inactivation of WT1 in this embryonic cancer (Ruteshouser and Huff, 2004). Some mutations result in the loss of genetic material leading to a truncated protein. A C-terminal deletion form of c-MYB was detected in a CML (chronic myeloid leukaemia) patient. Subsequent *in vitro* studies have indicated that truncated c-MYB displays enhanced transcriptional activity and is associated with B or T cell lymphomas in murine and bovine models (Introna and Golay, 1999). Gene amplification is another mutational process associated with tumourigenesis. For instance, amplification of N-MYC is one of the best studied alterations occurring in a sub-set of neuroblastoma patients and this alteration has been associated with greatly diminished survival probability (Seeger et al., 1985). Furthermore, a frequently observed mode of oncogenic activation involves chromosome translocations. Translocations occur during the transfer of a segment of one chromosome to another and therefore they may be reciprocal. A translocated gene can be placed under the control of different regulatory elements resulting in altered gene expression patterns. Such an example is the transcription factor c-MYC whose

coding sequence has been found in juxtaposition to the highly active immunoglobulin μ regulatory sequence in Burkitt's leukaemia (Taub et al., 1982). More commonly, translocations generate chimeric proteins, which consist of a fusion between parts of two genes. These hybrid proteins may have novel properties and frequently occur in leukaemia (Knudson, 2000). However, fusion genes were also observed in other malignancies. For instance, the paediatric primitive neuroectodermal tumour Ewing sarcoma is characterised by a specific translocation generating the EWS/FLI-1 hybrid. Whereas EWS/FLI-1 has the potential to transform cells, the transcription factor FLI-1 does not possess this ability and is a less potent transcriptional activator than its fusion protein counterpart (May et al., 1993).

1.1.5.2 Aspects of transformation

A plethora of different cancer types exists, but independently of their origin all cells have the same fundamental requirements. Initial genetic lesions provide cells with growth advantage enabling the selection of clones that harbour additional alterations in their genome. The accumulation of mutations ultimately leads cells to overcome boundaries that prevent transformation. These boundaries are all based on a few principles, which may be evaded in different ways depending on the nature of the genetic lesion. In order to adopt a malignant phenotype, cells need to acquire self-sufficiency in growth signals, insensitivity to growth-inhibiting signals, evasion of apoptosis, limitless replicative potential, sustained angiogenesis as well as tissue invasion and metastasis (Hanahan and Weinberg, 2000). These fundamental principles are briefly outlined in the following sections. Additionally, the role of transcription factors in these processes is highlighted with special emphasis on B-MYB, which is the main subject of this thesis.

1.1.5.2.1 Cell cycle progression is regulated by growth factors and inhibitors

The cell cycle regulatory machinery is the main pathway that controls normal cell growth and differentiation by integrating pro- and anti-proliferative signals. As a consequence, dysfunctional cell cycle regulation can lead to uncontrolled cell growth and cancer (Hanahan and Weinberg, 2000).

The cell cycle is a tightly regulated process consisting of many layers of regulation. A family of proteins called cyclin dependent kinases (Cdks) plays a crucial role in driving the cell through the cycle. Cdk activation requires the association with short lived proteins called cyclins and is further modulated by specific phosphorylation and dephosphorylation events at particular residues (Yam et al., 2002). The presence of the different cyclins varies during the cell cycle due to tight regulation of protein synthesis and degradation, therefore allowing only a limited window of Cdk activation. Transition through the cell cycle is orchestrated by the assembly and activation of consecutive cyclin/Cdk complexes, which in turn control cycle progression by phosphorylation of selected target proteins (Ekholm and Reed, 2000).

D-type cyclins reach maximal expression during early to mid-G1 in response to mitogenic stimulation, thereby providing a link between mitogen signalling and cell cycle progression. Once synthesised, the D-type cyclins consisting of cyclin D1, D2 and D3 form active complexes with both Cdk4 and Cdk6 to regulate G1-phase progression. Hence, D-type cyclins play an important role in the G1-phase of the cell cycle at which cells become committed to the next mitotic cycle (Ekholm and Reed, 2000). Cyclin E accumulates close to the G1/S border and, together with Cdk2, is important for transition through this phase by mediating phosphorylation of target proteins in late G1 (Ekholm and Reed, 2000). Cyclin A is synthesised after cyclin E and the expression levels peak in G2/M. Interestingly, cyclin A can associate with Cdk1 and Cdk2 and has functions in both S-phase and mitosis. During S-phase, cyclin A has been implicated in the control of DNA replication and many substrates of cyclin A are components of the DNA replication machinery (Yam et al., 2002). Cyclin B is produced following the induction of cyclin A during G2 and exclusively binds Cdk1. Together they constitute the principal mitotic complexes that regulate G2/M progression. It was proposed that cyclin A functions before cyclin B and controls its half life. Once cyclin B/Cdk1 is activated, the cyclin A/Cdk2 function is no longer required and cyclin B/Cdk1 drives mitosis exit (Yam et al., 2002).

Cyclin-dependent kinase inhibitors (CKI) constitute an additional layer of regulation. The Cip/Kip family is composed of $p21^{WAF1/CIP1}$, $p27^{KIP1}$ and $p57^{KIP2}$ and they can bind to and inhibit cyclin D/Cdk4/6 as well as cyclin E/A/Cdk2 complexes. The INK4 family of CKIs comprises p15, p16, p18 and p19 and they inhibit the association of Cdk4 and Cdk6 with cyclins. CKIs accumulate in response to growth inhibitory signals, such as cytokines or upon induction of differentiation or DNA damage (Ekholm and Reed, 2000).

A typical cyclin/Cdk target is the retinoblastoma protein pRb and the other family members p107 and p130. All pRb members bind to proteins belonging to the E2F family of transcription factors, whereby they inhibit E2F-mediated transactivation (Attwooll et al., 2004; Macaluso et al., 2006). Notably, the pRb family proteins display different expression patterns at various stages of the cell cycle and together they contribute to the regulation of E2F-responsive genes (Macaluso et al., 2006).

E2F family members form active DNA binding complexes with either DP1 or DP2. Although the DP subunit is critical for activity, the functional specificity of the E2F/DP complex is determined by the E2F subunit (Attwooll et al., 2004; Macaluso et al., 2006).
E2F-1, E2F-2, and E2F-3 are considered as the activating E2Fs and transactivate target genes important for G1/S transition. Ectopic expression of these E2F family members is sufficient to induce quiescent cells to enter S-phase. Interestingly, the E2F-3 locus encodes for the transcriptional activator E2F-3a and E2F-3b, which acts as a transcriptional repressor. E2F-4 and E2F-5 are poor transcriptional activators and they were proposed to be important for transcriptional repression. Moreover, they were attributed an important function in cell cycle exit and differentiation. E2F-6 and E2F-7 are both transcriptional repressors. E2F-6 is thought to play a role in quiescence and E2F-7 may regulate a subset of E2F target genes (Attwooll et al., 2004).

Transcriptionally activating E2F-1, E2F-2 and E2F-3 preferentially form complexes with pRb. In contrast, p107 as well as p130 mainly associate with the transcriptionally repressing members E2F-4 and E2F-5 (Macaluso et al., 2006). Upon mitogenic stimulation pRb members become phosphorylated by cyclin/Cdk

complexes and as a consequence they release E2Fs in order to activate target genes. In this manner, the E2Fs participate in both repression and activation of E2F-responsive genes. Importantly, E2Fs control the timely expression of many genes that are required for DNA replication and cell cycle control (Attwooll et al., 2004).

Untransformed cells strictly depend on growth signals allowing them to move from a quiescent into an active proliferative state. . Diffusible growth factors, extracellular matrix components and adhesion molecules provide such signals. In contrast, the requirement for growth factors is less pronounced in tumour cells. This is particularly relevant as during the course of tumour mass growth, cancer cells have to cope with periods of low nutrient and growth factor availability (Hanahan and Weinberg, 2000). Hence, during the process of transformation cancer cells evolve different mechanisms to evade dependence on growth promoting factors and these mechanisms might involve aberrant regulation of transcriptions factors. For instance, overexpression of the Id-1 transcription factor is related to progression of prostate cancer and elevated expression levels are frequently found in androgen independent tumours. Importantly, it was shown that ectopic expression of Id-1 reduces the dependence on androgen in prostate cancer cell lines (Ling et al., 2004). Expression of the transcription factor c-MYC is elevated in different human cancers and overexpression of c-MYC was shown to render cells growth factor independent (Fuhrmann et al., 1999). B-MYB is another transcription factor that when ectopically expressed is associated with a decreased dependence for growth factors (Sala and Calabretta, 1992; Grassilli et al., 1999). Moreover, constitutive expression of B-MYB counteracts cell cycle arrest of serum deprived BALB/c 3T3 fibroblasts (Sala and Calabretta, 1992).

Within a normal tissue, multiple anti-proliferative signals maintain tissue homeostasis by forcing cells into quiescence or differentiation. Cancer cells must circumvent these inhibitory signals in order to proliferate continuously (Hanahan and Weinberg, 2000). One common strategy of cancer cells to evade differentiation is to enhance the expression of transcription factors that are crucial regulators of cell proliferation and differentiation such as the MYC family members. For example, N-MYC amplification in neuroblastoma cells is associated with impaired differentiation capacity as these cells were shown to differentiate in the absence of

differentiation agents when N-MYC is silenced (Kang et al., 2006). Alternatively, transcription factor silencing provides another mechanism allowing escaping anti-proliferative signals. SMAD-4 transduces growth inhibitory signals induced by TGFβ signalling on the transcriptional level. Reduction of SMAD-4 expression frequently occurs in oesophageal adenocarcinoma and a variety of mechanisms were found to be responsible for SMAD-4 reduction in this type of cancer (Onwuegbusi et al., 2006). Notably, anti-proliferative signals induced by TGF-β, p53 or p107 negatively regulate B-MYB, suggesting that the presence of B-MYB might interfere with cellular quiescence (Lin et al., 1994; Satterwhite et al., 1994; Sala et al., 1996b). Importantly, constitutive expression of B-MYB has also been shown to counteract anti-proliferative signals by avoiding G1 growth arrests imposed by p53, p107 or $p57^{KIP2}$ (Lin et al., 1994; Sala et al., 1996a; Joaquin et al., 2002; Joaquin and Watson, 2003b). Moreover, B-MYB is downregulated during neuroblastoma differentiation and when overexpressed inhibits differentiation of these cells (Raschella et al., 1995; Pagnan and Sala, 2003).

1.1.5.2.2 Cell survival

Enhanced cell proliferation is not the only mechanism leading to a cell population expansion, as a decreased rate of cell death can also contribute to an elevated number of cells. Hence, it was proposed that acquired resistance towards cell death applies to most types of cancer (Hanahan and Weinberg, 2000).

In a normal tissue, apoptosis or programmed cell death is a physiological process, which enables the removal of excess, defective or damaged cells during development and maintenance of tissue homeostasis. Repression of apoptosis allows cell survival under circumstances that would normally elicit an apoptotic response such as the absence of growth factors, anti-survival signals or immune cell based cytotoxicity. Furthermore, a faulty apoptotic pathway has particularly severe effects during cancer therapy as defects in apoptosis attribute to the resistance to chemo- and radiation treatments (Tamm et al., 2001). Consequently, similar to perturbed regulation of cell cycle progression, cellular resistance to apoptosis allows the accumulation of further genetic lesions and thus contributes to the generation of cancer (Tamm et al., 2001).

Apoptosis is a firmly regulated process and the apoptotic machinery contains sensor components, which include cell surface as well as intracellular signalling molecules that activate death pathways (Green and Kroemer, 2005). The effector components of the apoptotic pathway comprise a set of proteases called caspases. Caspases are a family of single chain proteins, which exist in an inactive form in the cytoplasm and their activation involves proteolytic cleavage (Tamm et al., 2001). Caspases-8, -9 and -10 are so called initiator caspases and they are triggered by the activation of death receptors or by cytochrome c release. Initiator caspases activate an array of downstream effector caspases, such as caspase-3, -6 and -7, which in turn act by cleaving intracellular substrates (MacFarlane, 2003). A further level of regulation is introduced by inhibiting caspases through the action of proteins such as cFLIP or through a family of proteins called inhibitors of apoptosis that consist of members like IAP, XIAP and survivin (Tamm et al., 2001).

The intrinsic apoptotic pathway is induced in response to several stress situations such as irradiation, cytotoxic drugs or growth factor withdrawal. Such stress conditions trigger mitochondrial disruption and the release of cytochrome c, which is the critical event for the induction of the intrinsic apoptotic pathway (MacFarlane, 2003; Green and Kroemer, 2005). Cytochrome c release from the mitochondrion is regulated by the Bcl-2 family members, which share one or more Bcl-2 homology domains (Green and Kroemer, 2005). Bcl-2 family members are important regulators of cell survival and they possess antagonistic functions. Pro-survival members like Bcl-2, Bcl-x_L, MCL1 and A1 can associate with pro-apoptotic Bcl-2 family members such as Bax, Bak, Bim, Bid, Noxa, Puma and Bad. The release of cytochrome c depends on whether the balance is shifted to the anti- or pro-apoptotic Bcl-2 members. In response to apoptotic stimuli, Bax and Bak undergo a conformational change, oligomerise and disrupt the outer mitochondrial membrane (Tamm et al., 2001). Cytoplasmic cytochrome c binds to APAF1 and the resulting complex associates with procaspase-9, thus forming the apoptosome. Complex formation induces autocatalytic processing of caspase-9 and this in turn elicits the caspase activation cascade ultimately leading to apoptosis (Tamm et al., 2001).

On the other hand, the extrinsic apoptotic pathway is elicited upon the binding of ligands to death receptors, which in turn initiates the caspase cascade. Death receptors belong to the TNF receptor family and the most prominent members are FAS (CD95, Apo1), TNFR1 (p55, CD120a) and TRAIL-R1 with their cognate ligands (Tamm et al., 2001). Ligand binding induces receptor trimerisation and recruitment of the Fas-associated death domain (FADD) and other adaptor proteins to the cytoplasmic region of the receptor. This allows recruitment and activation of caspase-8 and -10, which again triggers the caspase activation pathway and ultimately results in apoptosis (MacFarlane, 2003; Green and Kroemer, 2005).

Cancer cells have been shown to employ a wide variety of mechanisms to evade apoptosis by modulating the expression levels of pro- as well as anti-apoptotic Bcl-2 family members, caspases and their inhibitors through deletion- and frame shift mutations (Tamm et al., 2001). Deregulated expression of transcription factors has been implicated in cell survival by directly affecting the expression of key players of the apoptotic machinery. An important example is the tumour suppressor p53, which is inactivated in a majority of cancers. p53 mainly regulates apoptosis through direct transcriptional induction of target genes such as Bax, Noxa, Puma and Bid in response to DNA damage (Haupt et al., 2003). Accumulating evidence suggests that B-MYB is an important modulator of stress situations as B-MYB confers protection against various insults including genotoxic drugs, thermal injury and DNA damage (Grassilli et al., 1999; Cervellera et al., 2000; Ahlbory et al., 2005; Santilli et al., 2005). It was proposed that ectopic expression of B-MYB can counteract the induction of the apoptotic pathway by increasing the transcription of anti-apoptotic target genes such as Bcl-2 or ApoJ/clusterin (Grassilli et al., 1999; Cervellera et al., 2000; Lang et al., 2005; Santilli et al., 2005).

1.1.5.2.3 Limitless replicative potential

Uncoupling from growth and apoptotic signals is insufficient for the establishment of a tumour as mammalian cells have a limited life span. Therefore, the restrictions imposed to ensure a limited life span need to be circumvented for a tumour to prosper (Hanahan and Weinberg, 2000).

After a limited number of cell divisions, normal cells cease to proliferate and die in a process called senescence. Cell doublings are monitored through the decrease in the length of telomeres. Telomeres are specialised structures occurring at the chromosome ends. They consist of repetitive DNA sequences and are capped by telomere binding proteins (Horikawa and Barrett, 2003). While somatic cells do not contain sufficient telomerase, which is the enzyme that synthesises telomeric DNA, telomerase expression is upregulated in virtually all malignant cells thus preventing them from undergoing senescence (Hanahan and Weinberg, 2000; Dimri, 2005). Hence, malignant transformation is accompanied by telomerase activation and subsequent telomere maintenance (Horikawa and Barrett, 2003).
Telomere shortening or uncapping of the telomere ends elicits a DNA damage response similar to the signal that is triggered by double-strand DNA breaks. The DNA damage response induces growth arrest mediated through the p53 pathway (Dimri, 2005).
Apart from telomere shortening, certain cellular stresses, like the activation of oncogenes, can also induce senescence. Thus, senescence can be triggered by either telomeric as well as non-telomeric signals and the two tumour suppressor genes p53 and pRb were identified as principal regulators of senescence (Dimri, 2005).

In human cells, telomeric signals are mediated through the p53/ $p21^{Waf1/Cip1}$/pRb pathway and non-telomeric signals engage both the p53/ $p21^{Waf1/Cip1}$/pRb as well as the p16/pRb pathway. In contrast, in murine cells senescence is triggered by the ARF/p53/ $p21^{Waf1/Cip1}$/pRb pathway (Dimri, 2005). Enforced expression of p53 or $p21^{Waf1/Cip1}$ can induce a senescence-like phenotype and inactivation of p53 or $p21^{Waf1/Cip1}$ can lead to complete abrogation of senescence. Conversely, loss of pRb during senescence is sufficient for cell cycle entry and the reversal of cellular senescence (Dimri, 2005). Therefore, cellular senescence is a tumour suppressive mechanism and immortalisation is necessary for cancer cells to thrive (Horikawa and Barrett, 2003).

Telomerase activity is mainly regulated through the transcriptional control of the catalytic subunit of telomerase, which is called the human telomerase reverse transcriptase (hTERT). Accumulating evidence suggests that several transcription factors, including proto-oncogenes such as c-MYC and E2F-1 as well as tumour

suppressors like p53 and WT1 may control the transcription of hTERT (Horikawa and Barrett, 2003). *In vitro* experiments have shown that c-MYB has the potential to immortalise primary cells as murine foetal liver cells transduced with c-MYB could be cultured in methylcellulose and further maintained in liquid culture (Ferrao et al., 1995). While B-MYB overexpression on its own appears to be unable to immortalise primary cells, B-MYB was shown to immortalise MEFs in combination with activated Ras (Masselink et al., 2001).

1.1.5.2.4 Angiogenesis

Oxygen supply is crucial for cell survival and solid tumours most often develop regions of hypoxia during the course of tumour mass growth. In order to ensure oxygen supply, tumours display angiogenic characteristics by producing angiogenesis-initiating factors, thus encouraging the growth of blood vessels (Ferrara and Kerbel, 2005). In the absence of blood supply, a tumour can grow to about 1-2 mm in diameter before it requires the generation of new blood vessels (Chin et al., 2005).

In an embryo, vessels are constantly being formed until the cardiovascular system is fully developed, whereas in adults, angiogenesis only occurs during tissue repair and wound healing (Chin et al., 2005). The process of angiogenesis is a tightly controlled event and blood vessel formation is initiated by soluble factors and their cognate receptors (Hanahan and Weinberg, 2000). Vascular endothelial growth factor (VEGF) family members and their cognate receptors are the most critical players of vascular formation and they are important in the initiation as well as development of immature vessels. VEGF is able to induce vascular permeability and vascular endothelial cell proliferation. Subsequently, endothelial cells differentiate within a previously blood vessel-devoid tissue and then aggregate to form a tubular network (Yancopoulos et al., 2000). Afterwards, smooth muscle cells (SMCs) cover the endothelial cells and they stimulate the production of extracellular matrix components, which stabilise the vessel walls (Carmeliet, 2000).

Tumours may induce angiogenesis by increasing the abundance of angiogenesis inducers as tumour cells were found to produce VEGF family members and other

angiogenic factors such as bFGF, angiopoietins and interleukin-8 (Ferrara and Kerbel, 2005). One common strategy of cancer cells to increase the abundance of angiogenesis inducers may involve altered gene transcription. An example is the hypoxia inducible factor 1 (HIF-1), which binds to the hypoxia-responsive element in the regulatory regions of hypoxia-inducible genes. HIF-1 regulates the expression of genes associated with adaptation to reduced oxygen levels such as the VEGF family members (Ferrara and Kerbel, 2005). Accordingly, overexpression of the HIF-1 subunit α is associated with increased cell growth and metastatic potential. Increased expression of HIF-1α is found in the majority of prostate tumours and one mechanism leading to increased expression levels could be attributed to gene amplification, as the HIF-1α gene was found to be amplified in 36% of primary prostate tumours (Saramaki et al., 2001).
While B-MYB expression is highest in proliferating SMCs, ectopic expression of B-MYB is unable to induce proliferation of SMCs (Marhamati et al., 1997). Therefore, deregulated expression of B-MYB does not appear to influence the lining of SMC along the primitive blood vessel. However, overexpression of B-MYB was found to negatively affect the promoter activity of several extracellular matrix components like the type I α1(I) and α2(I) as well as the type V α2(V) collagen chains in SMCs (Marhamati and Sonenshein, 1996; Kypreos et al., 1999). Yet, whether the ability of B-MYB to regulate expression of extracellular matrix components might influence vessel formation within a tumour mass remains subject to speculation.

1.1.5.2.5 Invasion and metastasis

Cellular invasion is important for normal biological processes like development, immune response and wound healing. However, the ability to invade and to metastasise enables cancer cells to escape the primary tumour mass and settle at new sites in the body. To colonise new territory, cancer cells need to invade the surrounding stroma, enter the blood stream, which transports them to distant sites from where they may infiltrate a particular tissue and form a secondary tumour mass (Meyer and Hart, 1998). The capacity of malignant tumours to metastasise is mainly responsible for their lethality. Metastatic tumour spread accounts for about

90% of all human cancer deaths (Meyer and Hart, 1998; Hanahan and Weinberg, 2000).

Key processes implicated in tissue invasion and metastasis involve the production of proteolytic enzymes capable of degrading the stroma as well as altered expression of cell surface adhesion proteins (Meyer and Hart, 1998; Chin et al., 2005).
Cancer cells are anchored to the primary tumour site through cell to cell or cell to substrate interactions. Members of the cadherin family predominantly mediate cell-cell interactions. Cadherins are transmembrane adhesion proteins whose cytoplasmic domains interact with the cytoskeleton via the catenin family members. Importantly, loss or reduction of E-cadherin was observed in various cancer types (Meyer and Hart, 1998). In metastatic cells, alterations also occur in the expression pattern of integrin receptors allowing neoplastic cells to migrate along the underlying substrate. While integrins are transmembrane receptors that function predominantly by linking cells to the extracellular matrix (ECM), they also influence cellular migration as well as induce signalling transduction cascades (Meyer and Hart, 1998).

Furthermore, matrix degrading proteases are important to overcome the barrier imposed by the ECM that forms the connective tissue around the tumour (Chin et al., 2005). Matrix metalloproteinases (MMPs) are associated with the degradation of extracellular matrix components. As MMPs occur in an inactive form they require proteolytic cleavage themselves in order to become active (Chin et al., 2005). Degradation of structural components of the ECM by MMPs facilitates cell migration and their action regulates the tumour microenvironment. While cancer cells synthesise some MMPs, they are predominantly generated by stromal cells. However, cancer cells may stimulate the surrounding stromal cells to synthesise MMPs (Egeblad and Werb, 2002; Chin et al., 2005). As a consequence, the expression and activation of MMPs is increased in most human cancers and with a few exceptions, increased expression and activation of MMPs correlates with advanced tumour stage, increased invasion and metastasis behaviour and poor prognosis (Egeblad and Werb, 2002).

Aberrantly activated or expressed transcription factors may directly contribute to cell motility and invasion. For example, p53 was shown to negatively affect gene expression of MMP1 and MMP13 (Egeblad and Werb, 2002). The dimeric AP-1 transcription factor complex has oncogenic potential as its components c-Jun and c-Fos may facilitate and are required for transformation. Importantly, AP-1 complexes were shown to activate the expression of various MMPs (Ozanne et al., 2007). No evidence exists as yet indicating that B-MYB might promote invasion or metastasis. Even though B-MYB overexpressing MEFs escape activated Ras-induced senescence, only a subset of these cells were able to grow in soft agar, indicating that further genetic lesions were required to allow anchorage-independent growth (Masselink et al., 2001). Conversely, the B-MYB target gene ApoJ/clusterin was shown to inhibit invasion of neuroblastoma cells *in vitro* (Santilli et al., 2003). It would be interesting to further address whether B-MYB might possess metastasis suppressive capabilities through the activation of ApoJ/clusterin.

Overall, transcription factors can modulate all aspects important for transformation and thus when abnormally expressed or activated transcription factors contribute to the development of cancer. Moreover, B-MYB, which is the main subject of this thesis, has been shown to possess characteristics that suggest an important role in different aspects of tumourigenesis. The characteristics of B-MYB will be discussed in greater detail in the next chapter.

1.2 B-MYB

1.2.1 The MYB family of transcription factors

The MYB proteins form a family of multidomain transcription factors in mammals, avian, insects, yeast and plants (Oh and Reddy, 1999). The N-terminal DBD shows the highest degree of conservation and the vertebrate MYB family members share about 90% sequence identity in this region. The DBD is conserved throughout evolution and is especially common in transcription factors of plants (Ito, 2005). In higher vertebrates three family members have been identified (c-MYB, A-MYB and B-MYB) and all activate transcription from responsive promoters (Oh and Reddy, 1999).

c-MYB is the cellular progenitor of its truncated, viral counterpart v-MYB. v-MYB is carried by the avian retroviruses AMV and E26, which are capable of transforming erythroid and myeloid cells (Oh and Reddy, 1999). The discovery of c-MYB led to the isolation of A-MYB and B-MYB from human sources by low-stringency cross-hybridisation with a c-MYB DNA probe (Nomura et al., 1988).

Human B-MYB mRNA codes for a protein of 704 amino acids with an apparent molecular mass of 93 kilodaltons (Oh and Reddy, 1999). Interestingly, a less abundant, alternative B-MYB transcript lacking exon 9A was detected in a number of cells (Kamano et al., 1995). It was reported that this splice form is transcriptionally inactive and competes with full-length B-MYB, thereby inhibiting transactivation induced by the full-length protein (Horstmann et al., 2000a). However, it still remains to be proven whether an endogenous truncated B-MYB protein normally occurs in cells and consequently whether the proposed antagonistic effect of the two splice variants constitutes a naturally occurring regulatory mechanism.

1.2.2 Expression patterns of the vertebrate MYB family members

Vertebrate MYBs display distinct cell type-specific expression, whereby some tissues may express more than one MYB family member (Oh and Reddy, 1999). Studies aimed at elucidating the expression pattern of the MYB family members revealed that unlike c-MYB and A-MYB, which occur in a more tissue restricted manner, B-MYB is more broadly expressed.

Although c-MYB is mainly expressed in proliferating, primary haematopoietic cells and in various leukaemia cell lines (Westin et al., 1982; Golay et al., 1991), the c-MYB transcript was also detected in non-haematopoietic tumours (Torelli et al., 1987; Raschella et al., 1999). In addition, c-MYB occurs in some untransformed, non-haematopoietic tissues, including the developing brain, thymus, lung and epithelial cells of the colon (Torelli et al., 1987; Thiele et al., 1988; Takahashi et al., 1995; Tashiro et al., 1995).
A study with mice harbouring a disrupted c-MYB gene indicated that c-MYB is essential for proliferation of immature haematopoietic cells. Initially, the development of these mice appeared normal suggesting that c-MYB is not required for early development. However, at day 15 of gestation homozygous mice were severely anaemic and died due to defective foetal liver haematopoiesis (Mucenski et al., 1991).

A-MYB was only detected in testis and peripheral blood leukocytes from a panel of normal human tissues (Takahashi et al., 1995). Murine A-MYB expression was abundant in the developing central nervous system and the urogenital ridge. In the adult mouse, A-MYB could be detected during the early stages of sperm cell differentiation and in proliferating B lymphocytes located in the spleen (Trauth et al., 1994). Despite A-MYB expression being restricted to certain tissues in a healthy organism, the mRNA was identified in a broad range of cancer cell lines including kidney, colon as well as lymphoma cell lines (Nomura et al., 1988).
In contrast to the other MYB family members, disruption of a functional A-MYB gene is not embryonic lethal as homozygous embryos develop normally until birth. However, homozygous male mice were infertile due to arrested spermatogenesis

and although female homozygous mice were fertile, they were unable to wean their pups. These findings indicate that A-MYB is required for spermatogenesis and development of breast tissue after pregnancy (Toscani et al., 1997).

B-MYB is broadly expressed in different transformed cell lines including leukaemia, neuroblastoma, stomach carcinoma and colon carcinoma cells (Nomura et al., 1988) as well as in proliferating primary cells (Golay et al., 1991).
In mouse embryos, B-MYB expression was found in all proliferating tissues and consequently absent in terminally differentiated cells (Sitzmann et al., 1996). In the adult mouse, B-MYB mRNA expression is high in thymus, spleen and testis. Moderate B-MYB levels occur in the small intestine as well as in the colon and low levels are present in ovary, peripheral leukocytes, placenta, lung and pancreas (Tashiro et al., 1995).
Considering this broad expression pattern, it is not surprising that B-MYB deficient mice die very early during development, around embryonic day 4.5 – 6.5. *In vitro* propagation of B-MYB deficient blastocysts indicated that depletion of B-MYB impairs defective proliferation of the inner cell mass (ICM). The trophoectoderm was more resistant to B-MYB ablation than the ICM, which may be more sensitive because of its rapid cell division capacity (Tanaka et al., 1999).
Whereas c-MYB and A-MYB are not, the B-MYB transcript is highly expressed in embryonic stem (ES) cells, making B-MYB the exclusive MYB family member in these cells (Tanaka et al., 1999). Furthermore, ES cells with homozygously disrupted B-MYB could not be generated, indicating that B-MYB is essential for their survival (Tanaka et al., 1999).
Interestingly, inhibition of MYB target gene expression with a dominant interfering MYB construct caused colonies of these ES cells to disperse into single cells that detached from the substrate. This indicates that blocking B-MYB transactivation activity leads to defective cell adhesion. In agreement, inhibition of B-MYB target gene induction resulted in reduced cell surface expression of β1-integrin and E-cadherin, despite the respective mRNAs remained unchanged (Iwai et al., 2001).

1.2.3 Evolutional development of the MYB family: important lessons from an inconspicuous organism

The fact that higher vertebrates encode three different MYB proteins and that their expression patterns vary considerably, suggests that each MYB member has specific and distinct functions. This notion is supported by the observation that although B-MYB is ubiquitously expressed in proliferating cells, judging from the phenotypes of c-MYB or A-MYB deficient mice, B-MYB is functionally unable to compensate for the loss of the other MYBs. This strongly indicates that the MYB family members are not functionally redundant and hence it was proposed that c-MYB and A-MYB might have arisen by gene duplication from a common ancestor (Davidson et al., 2005).

In contrast to vertebrates, invertebrates such as the fruit fly *Drosophila melangolaster* contain only one single MYB gene, called Dm-MYB (Lipsick et al., 2001). Absence of Dm-MYB causes failure of larval lymph gland development and proliferation of haemocytes, which are specialised cells involved in haematopoiesis. Genetic rescue experiments indicated that vertebrate B-MYB, but not c-MYB or A-MYB, can complement these defects and therefore vertebrate B-MYB and DM-MYB were proposed to be functional orthologues. Thus, a common ancestor of the MYB family presumably was a gene closely related to the vertebrate B-MYB. Consequently, c-MYB and A-MYB might have arisen from this common ancestor gene and over time they have acquired alterations in their coding regions that resulted in new gene functions (Davidson et al., 2005).

1.2.4 B-MYB structure

Functionally, the MYB proteins can be separated into three distinct domains: a DBD, a transactivating domain and a regulatory domain (Figure 1-3). The DBD is highly conserved among the three vertebrate MYBs and accordingly the MYB family members are able to recognise similar DNA sequences (Mizuguchi et al., 1990; Oh and Reddy, 1999). Whereas the DBDs display high sequence homology, the remaining domains are considerably less conserved. The presence of less conserved regions supports the notion that c-MYB, A-MYB and B-MYB might have independent functions in their target cell types (Oh and Reddy, 1999).

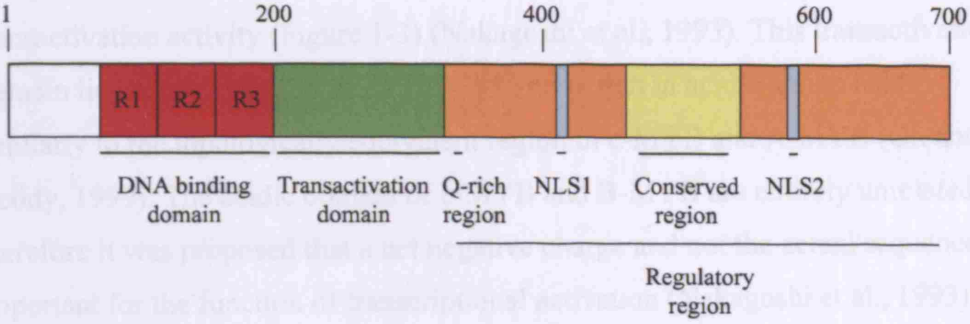


Figure 1-3 Schematic domain organisation of the B-MYB protein
The N-terminal DBD consists of three regions termed R1, R2 and R3, which is followed by the acidic transactivation domain. The C-terminal part of B-MYB regulates the transactivation activity. This portion harbours a region rich in glutamine (Q-rich region) a conserved region and two nuclear localisation signals (NLS). The numbers above the drawing indicate the amino acid positions.

1.2.4.1 The DNA binding domain

The N-terminal DBD of B-MYB consists of three imperfect tandem repeats of some 50 amino acids (Figure 1-3) and each repeat contains three conserved tryptophans. The tryptophans contribute to the hydrophobic core that constitutes a HTH-related structure, which mediates contacts with the DNA (McIntosh et al., 1998; Oh and Reddy, 1999).

Interestingly, *in vitro* DNA binding activity cannot be detected under standard EMSA conditions with the full-length B-MYB protein, yet DNA binding can be observed upon deletion of the C-terminal portion of the protein, suggesting that the B-MYB carboxy-terminus prevents the DBD from binding to DNA (Watson et al., 1993). However, ChIP (chromatin immunoprecipitation) analysis allowed the detection of endogenous B-MYB associated with promoters of its target genes (Tanno et al., 2002; Santilli et al., 2005). These contrasting results may reflect the nature of the different techniques used to study B-MYB DNA binding.

1.2.4.2 The transactivation domain

A region downstream of the DBD of B-MYB was shown to be required for transactivation activity (Figure 1-3) (Nakagoshi et al., 1993). This transactivation domain lies between amino acids 205-338 and is rich in acidic amino acids, similarly to the topologically equivalent region in c-MYB and A-MYB (Oh and Reddy, 1999). The acidic domain of c-MYB and B-MYB are entirely unrelated and therefore it was proposed that a net negative charge and not the actual sequence is important for the function of transcriptional activation (Nakagoshi et al., 1993). Additionally, a glutamine-rich region is located between amino acids 346-360. It was proposed that considering the transactivation function of glutamine-rich regions in other transcription factors, this region could contribute to the transcriptional activation of B-MYB (Nakagoshi et al., 1993).

1.2.4.3 The regulatory domain

The C-terminus downstream of the transactivation domain was suggested to negatively regulate the transactivation activity of B-MYB. This hypothesis is supported by the fact that C-terminal deletion of about 200 amino acids increases the transactivation potential of B-MYB (Ziebold et al., 1997; Lane et al., 1997; Bessa et al., 2001).

The B-MYB C-terminus comprises a conserved region (CR) between amino acids 468-545 (Figure 1-3), which is highly homologous among the MYB family members and deletion of this region negatively affects the B-MYB transactivation function (Nakagoshi et al., 1993; Tashiro et al., 1995; Lane et al., 1997). Interestingly, cotransfection of the CR decreased the transactivation potential of full-length B-MYB suggesting that the CR competes for co-factors required for B-MYB transactivation. Notably, GST-pull downs with the CR of B-MYB revealed that depending on the cell type CR-binding proteins vary (Tashiro et al., 1995). However, more recent experiments questioned whether the CR influences the transactivation potential of B-MYB. As reporter assays performed by Bessa et al. showed that a truncation mutant of B-MYB lacking the most C-terminal part as well as the CR did not abolish the transactivation potential (Bessa et al., 2001).

B-MYB is strictly localised to the nucleus and the CR is flanked by two nuclear localisation signals (NLSs) (Figure 1-3). The first NLS is located between amino acids 411-418 and the second is found in the region between amino acids 564-584. Initially it was observed that mutants lacking either NLS were still primarily expressed in the nucleus (Nakagoshi et al., 1993; Charrasse et al., 2000). However, another group generated a B-MYB mutant lacking the second NLS only and they found B-MYB expression in the nucleus as well as in the cytoplasm (Johnson et al., 2002). Even though these reports are conflicting regarding the function of the NLSs, a greater proportion of mutants lacking both NLSs were detected in the cytoplasm suggesting that the two NLSs act in a cooperative manner (Nakagoshi et al., 1993; Petrovas et al., 2003).

1.2.5 Regulation of B-MYB

B-MYB abundance and activation is tightly linked to the cell cycle. Transcription of B-MYB is regulated by different E2F complexes and activation of the B-MYB protein is achieved by cyclin-dependent phosphorylation, which in turn triggers degradation of B-MYB. Furthermore, interactions with binding partners fine tune the B-MYB activity by enhancing or repressing its transactivation potential. The combination of all levels of regulation ensures firm control over the production of abundant hyperactivated B-MYB in the S-phase of the cell cycle.

1.2.5.1 The B-MYB promoter and its transcriptional regulation

Early synchronisation experiments have shown that the B-MYB mRNA, as well as the translated protein, are growth regulated peaking in the early S-phase and starting to decline as cells leave the replication phase entering G2/M (Lam and Watson, 1993; Robinson et al., 1996).

Several studies have supported the notion that virtually all proliferating cell lines transcribe the B-MYB gene in the G1/S phase of the cell cycle, although lower but detectable levels of the B-MYB protein are also observed during the G0- and G2/M-phases of the cell cycle (Robinson et al., 1996). Practically all studies addressing the transcriptional regulation of B-MYB have been performed with cells

released from G0. Yet, this experimental approach does not explain whether B-MYB is directly induced by the addition of mitogens. However, B-MYB protein expression was also strongly enhanced during the S-phase of cells released from an M-phase arrest (Robinson et al., 1996).

Comparison of the human and murine 5'-untranslated regions immediately upstream of the coding region of B-MYB show significant nucleotide conservation. A conserved E2F binding site (CTTGGCG) at position -212 to -201 relative to the ATG codon in the murine B-MYB promoter has been implicated in transcriptional repression during G0/G1 as mutation of this site abolished inhibition of a reporter gene in quiescent cells (Lam and Watson, 1993; Rayman et al., 2002). EMSAs with nuclear extracts from HELA cells indicated that the B-MYB promoter E2F site interacts with both activating and repressing E2F complexes, including E2F-1/DP-1, E2F-3/DP-1 and E2F-4/DP-1 complexes (Figure 1-4) (Liu et al., 1996). B-MYB mRNA expression in quiescent MEFs from mice harbouring a mutated E2F site in the germ line was also significantly derepressed, but the B-MYB transcript was still induced following re-entry into the cell cycle suggesting that the promoter harbours additional growth regulated elements that act independently of the E2F binding site (Tavner et al., 2006).

Moreover, binding of repressive E2F complexes to the E2F site is strongly supported by an adjacent downstream repression site (DRS) located at -198 to -194 (GGAAA) (Figure 1-4) (Liu et al., 1996; Catchpole et al., 2002). Similar to the requirement for an intact E2F binding site, the B-MYB promoter was also found to be significantly derepressed in $p107^{-/-}/p130^{-/-}$ MEFs (Catchpole et al., 2002; Rayman et al., 2002). Consequently, EMSA and ChIP assays have revealed that the B-MYB promoter is bound by E2F-4/p107 and E2F-4/p130 complexes in quiescent cells only when both the E2F and DRS elements were intact (Catchpole et al., 2002; Rayman et al., 2002). Interestingly, while the B-MYB promoter is bound by both p107 and p130 it is not associated with pRb in asynchronously growing human T98G cells (Takahashi et al., 2000).

Additionally, in quiescent cells the B-MYB regulatory sequence is also associated with HDAC co-repressor complexes such as HDAC1 and mSin3B and to a lesser extent with HDAC2 and mSin3A, which presumably mediate transcriptional repression by keeping the promoter in a hypoacetylated state (Figure 1-4).

Experiments with NIH 3T3 cell lines comprising an integrated B-MYB promoter transgene showed that the E2F site is necessary for recruitment of HDAC complexes to repress the B-MYB gene following serum starvation (Rayman et al., 2002). Furthermore, p107 and p130 are presumably required for the presence of HDACs at the B-MYB promoter as HDACs are missing at the promoter in $p107^{-/-}$ /$p130^{-/-}$ MEFs. In summary, these findings indicate that repressive complexes hold the B-MYB promoter in a transcriptionally inactive state in G0/1 (Rayman et al., 2002).

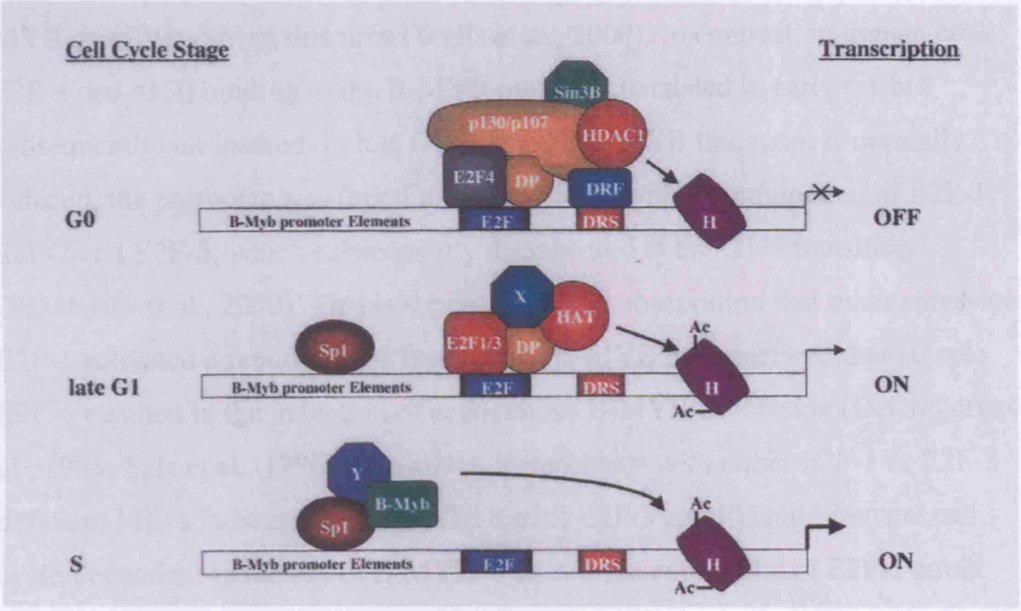


Figure 1-4 Transcription of B-MYB is governed by key players of the cell cycle
In quiescent cells, the B-MYB promoter E2F site and the downstream DRS element are bound by a repressor complex. This complex includes E2F-4, DP-1, p107, p130 and several other proteins involved in inhibiting transcription. In late G1, when pRb family members disappear from the complex, the E2F site is bound by E2F-1/3 and DP-1. The complex is now associated with proteins that possess HAT activity, conferring the promoter region to a transcriptionally active state and therefore initiating transcription. In S-phase, B-MYB transcription is fully derepressed. While the E2F site is unoccupied, Sp1 and B-MYB cooperate in compelling transcription of the B-MYB promoter. The illustration is from (Joaquin and Watson, 2003a).

While there are some discrepancies regarding the exact mechanism leading to induction of the B-MYB promoter, it is generally acknowledged that the associated E2F-4 complexes disappear from the B-MYB promoter when cells enter the cell cycle (Figure 1-4) (Zwicker et al., 1996; Wells et al., 2000; Takahashi et al., 2000). Moreover, footprinting experiments support the theory that the B-MYB promoter E2F site is unoccupied during the S-phase when B-MYB transcription is maximal (Zwicker et al., 1996).

In murine cells, E2F-4 binding was reported to be reduced at the G1/S-boundary and was completely absent during S-phase. Binding of p107 and p130 vanished prior to that of E2F-4 namely in late G1, indicating that free E2F-4 binds to the B-MYB promoter during this time (Wells et al., 2000). In contrast, in human cells E2F-4 and p130 binding to the B-MYB promoter persisted in early G1 but subsequently diminished. In late G1, when the B-MYB transcript is normally induced, the promoter was found to be associated with a combination of E2F-1, E2F-2 and E2F-3, which subsequently disappeared at the G1/S transition (Takahashi et al., 2000). This is supported by the observation that overexpression of E2F-1 activated a reporter gene fused to the B-MYB promoter and that ectopic E2F-1 resulted in the induction of endogenous B-MYB expression (DeGregori et al., 1995; Sala et al., 1996a). However, experiments with either E2F-1 or E2F-3 deficient MEFs indicated that only the loss of E2F-3 significantly impairs cell cycle-dependent induction of B-MYB. Yet, ectopic expression of E2F-1 could rescue the proliferation defect of E2F-3$^{-/-}$ MEFs, indicating that at least when overexpressed both proteins might show some functional redundancy (Humbert et al., 2000).

Interestingly, B-MYB gene activation coincided with increased regional acetylation of histones H3 and H4 in late G1, which was sustained during the S-phase (Takahashi et al., 2000). Thus, it is widely believed that loss of repressor complexes on the B-MYB promoter facilitates hyperacetylation of the surrounding nucleosomes, which in turn leads to gene activation (Rayman et al., 2002). Importantly, acetylated histone H3 was also found on the B-MYB promoter of quiescent MEFs from mice with a mutated E2F site, implying that deacetylation of the B-MYB promoter in quiescent cells is dependent on the presence of a repressive E2F complex (Tavner et al., 2006).

1.2.5.2 Post-translational modifications regulate B-MYB activity

1.2.5.2.1 B-MYB phosphorylation

Initially, a higher molecular weight form of the B-MYB protein was observed in synchronised murine cells in S-phase. As phosphatase treatment of the cell lysate reversed the electrophoretic mobility of B-MYB to the lower molecular weight form, it was suggested that B-MYB becomes hyperphosphorylated during the S-phase (Robinson et al., 1996). This assumption was corroborated by the fact that co-expression of cyclin A and Cdk2 induced this mobility shift and facilitated incorporation of radioactive phosphate into B-MYB (Robinson et al., 1996; Lane et al., 1997; Ziebold et al., 1997). Importantly, kinase assays showed that B-MYB can also be phosphorylated in cell free systems by cyclin A/Cdk2 complexes (Saville and Watson, 1998; Johnson et al., 1999). Furthermore, specific B-MYB phosphorylation during cell cycle progression was demonstrated, as a phosphorylated form of B-MYB was immunoprecipitated from S-phase but not from quiescent cells (Ziebold et al., 1997).
Notably, the electrophoretic mobility of B-MYB was also altered in human cells overexpressing cyclin A or cyclin E reminiscent of the phosphorylated form observed in murine cells (Sala et al., 1997). In contrast to cyclin A or cyclin E, a higher molecular weight form of B-MYB was not detectable in combination with cyclin B or cyclin D1 further supporting that B-MYB phosphorylation is a specific S-phase event (Sala et al., 1997; Ziebold et al., 1997).

Two-dimensional tryptic phospho-peptide analysis showed that cyclin A/Cdk2 phosphorylated similar B-MYB sites *in vitro* as well as *in vivo* and that these spot patterns coincided with the ones obtained from fibroblasts, which were synchronised in the S-phase of the cell cycle (Ziebold et al., 1997). Subsequent analyses using site directed mutagenesis followed by two-dimensional tryptic phospho-peptide mapping revealed that the following serines (S) and threonines (T) of murine B-MYB are cyclin A/Cdk2 substrates: T267, T408, T443, T447, T490, T497, T519, T522, T524 and S283, S343, S396, S424, S455, S581 (Saville and Watson, 1998; Bartsch et al., 1999; Johnson et al., 1999; Johnson et al., 2002). All

15 phosphorylation sites are conserved in human B-MYB, suggesting that this protein is also subjected to the same post-translational modifications at the corresponding sites (see amino acid sequence alignment Figure 3-14B).

The cyclin A/Cdk2 kinase activity was shown to specifically increase the transactivation function of B-MYB on several reporter constructs, indicating that phosphorylation may regulate the B-MYB transactivation capacity (Figure 1-5) (Lane et al., 1997; Sala et al., 1997; Ziebold et al., 1997; Saville and Watson, 1998; Johnson et al., 1999; Muller-Tidow et al., 2001; Bessa et al., 2001; Johnson et al., 2002). This observation is strongly supported by the fact that the B-MYB transactivation function was inhibited in the presence of a dominant negative form of Cdk2 (Bessa et al., 2001).

In addition to cyclin A, cyclin A1/Cdk2 complexes were also shown to increase phosphorylation of B-MYB and render it more transcriptionally active (Muller-Tidow et al., 2001). Cyclin A1 is specifically expressed in early embryonic tissues (Yam et al., 2002). In the adult, cyclin A1 shows a more restricted expression pattern than the ubiquitous cyclin A. Namely, cyclin A1 specifically occurs in testis and brain as well as in several myeloid leukaemia cell lines (Yang et al., 1997).

While mutant forms of B-MYB lacking single phosphorylation sites retained considerable levels of transactivation activity, simultaneous mutation of several phosphorylation sites significantly affected the B-MYB transactivation potential (Saville and Watson, 1998; Bartsch et al., 1999; Johnson et al., 1999). A B-MYB construct concomitantly mutated at 10 phosphorylation sites (mut10) still displayed residual transactivation activity, but transactivation was completely abolished when all 15 known phosphorylation sites were mutated (mut15) (Johnson et al., 2002). These findings suggest that the B-MYB transactivation activity is not considerably affected by a single phosphorylation site, but it is rather cumulatively enhanced by phosphorylation at several sites.

Paradoxically, it was observed that mutation of certain phosphorylation sites of B-MYB significantly increases DNA binding affinity, even though these mutant constructs less efficiently induce a reporter gene (Johnson et al., 1999). However,

other groups did not find any evidence that phosphorylation of B-MYB modulates its DNA binding affinity and this issue remains unresolved to date (Saville and Watson, 1998; Bessa et al., 2001).

Apart from phosphorylation, removal of the B-MYB C-terminus also increases its transactivation potential (Ziebold et al., 1997; Lane et al., 1997; Ansieau et al., 1997; Bessa et al., 2001). These findings suggest that the B-MYB transactivation activity is repressed by its C-terminus and that negative regulation by this region can be overcome by protein truncation or cyclin A/Cdk2 phosphorylation. A model that incorporates the two observations was proposed by Lane and colleagues whereby phosphorylation of B-MYB is believed to induce a conformational change in a manner that abrogates the suppressive effect of the C-terminal region (Lane et al., 1997).

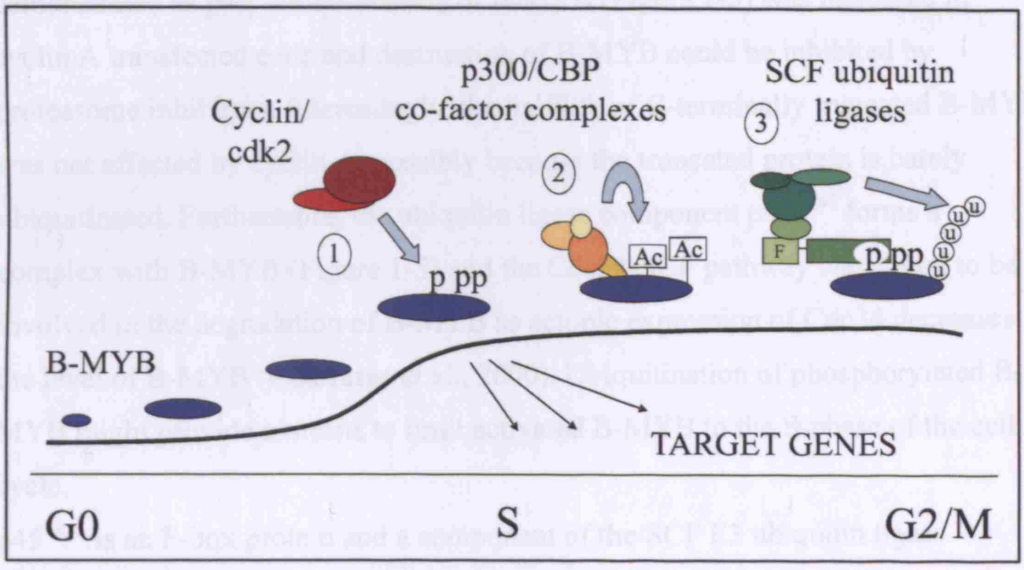


Figure 1-5 B-MYB is subjected to various post-translational modifications
B-MYB is transcriptionally regulated during the cell cycle and maximal expression levels peak in S-phase. 1) Phosporylation by cyclin A/Cdk2 during S-phase renders B-MYB more transcriptionally active on certain promoters. 2) Direct interaction allows p300 to acetylate B-MYB and thus enhances its transactivation potential. 3) Phosphorylation of B-MYB also induces its targeting to the proteasome by ubiquitination through the SCF pathway.

1.2.5.2.2 B-MYB acetylation

B-MYB has been further observed to be acetylated by p300 and the acetylation sites were mapped to the C-terminus (Figure 1-5) (Johnson et al., 2002; Schubert et al., 2004). Importantly, experiments with a mutant form of p300 that lacks HAT activity have revealed that transactivation of B-MYB is acetylation dependent (Schubert et al., 2004).

1.2.5.2.3 B-MYB ubiquitination

Co-expression of cyclin A does not only enhance the B-MYB transactivation activity, but also reduces B-MYB expression levels (Saville and Watson, 1998; Charrasse et al., 2000). Experiments measuring the protein half life confirmed that in the presence of cyclin A the B-MYB turnover was considerably increased. Involvement of the 26S proteasome pathway in the degradation process was demonstrated as poly-ubiquitination of B-MYB (Figure 1-5) was increased in cyclin A transfected cells and destruction of B-MYB could be inhibited by proteasome inhibitors. Interestingly, the half life of C-terminally truncated B-MYB was not affected by cyclin A, possibly because the truncated protein is barely ubiquitinated. Furthermore, the ubiquitin ligase component $p45^{Skp2}$ forms a complex with B-MYB (Figure 1-5) and the Cdc34-SCF pathway was found to be involved in the degradation of B-MYB as ectopic expression of Cdc34 decreases the level of B-MYB (Charrasse et al., 2000). Ubiquitination of phosphorylated B-MYB might provide a means to limit activated B-MYB to the S-phase of the cell cycle.

$p45^{Skp2}$ is an F-box protein and a component of the SCF E3 ubiquitin ligase complex, which functions in the destruction of a plethora of proteins. F-box proteins are interchangeable adaptors that link a core ubiquitin ligase with different substrates. B-MYB is presumably degraded in a strictly cell cycle dependent manner as $p45^{Skp2}$ is itself cell-cycle regulated at the transcriptional level (Harper and Elledge, 1999). SCFs are modular complexes that consist of proteins such as SKP1, CDC53, Cdc34 and one of a variety of F-box proteins (Harper and Elledge, 1999; Koepp et al., 1999).

1.2.5.3 Cooperation with co-factors

Gene transcription is not only regulated through the action of transcription factors but is rather a complex interplay between transcription factors and a plethora of co-factors that both support or hinder transcription by regulating the structure of the underlying DNA (Narlikar et al., 2002). Various co-repressors interact with transcription factors and they act by recruiting HDACs to target gene promoters (Jepsen and Rosenfeld, 2002).

As transcription of B-MYB responsive promoters can be greatly enhanced in the presence of the HDAC inhibitor trichostatin A, it was suggested that the existence of HDACs at promoters of B-MYB target genes may hinder their transcriptional activation (Li and McDonnell, 2002). Indeed, the B-MYB transactivation activity was repressed in the presence of the co-repressors BRAM1, a splice variant of BS69 (Masselink et al., 2001) and expression of dominant negative forms of N-CoR and SMRT increased the transactivation ability of B-MYB (Figure 1-6) (Li and McDonnell, 2002). Consequently, B-MYB was shown to physically interact with these co-repressor proteins (Figure 1-7) (Masselink et al., 2001; Li and McDonnell, 2002). While it has been demonstrated that BS69 binds to the C-terminus of B-MYB (Masselink et al., 2001) it is not clear at present whether N-CoR and SMRT interact with B-MYB through the same domain (Masselink et al., 2001; Li and McDonnell, 2002). Yet, the association between B-MYB and N-CoR is reduced in the presence of overexpressed cyclin A/Cdk2, suggesting that phosphorylation blocks the interaction between B-MYB and N-CoR (Li and McDonnell, 2002).

On the other hand, B-MYB binds to and is transcriptionally stimulated by the general co-activator proteins p300 and CBP (Figure 1-6 and Figure 1-7) (Bessa et al., 2001; Li and McDonnell, 2002; Johnson et al., 2002; Schubert et al., 2004). Both proteins possess intrinsic HAT activity and have been proposed to serve as platforms for the recruitment of additional co-factors as well as to link transcription factors to the PIC (Lee and Young, 2000).

While the ability of CBP to stimulate B-MYB transactivation is dependent on B-MYB being phosphorylated, physical interaction between CBP and B-MYB is not affected by the B-MYB phosphorylation status (Bessa et al., 2001).

The ability of p300 to act as a co-activator of B-MYB was found to depend on the promoter context (Bartusel et al., 2005). The primary interaction site with p300 was mapped to the B-MYB transactivation domain (Schubert et al., 2004) and the acetylation of B-MYB appears to depend on physical interaction with p300 (Johnson et al., 2002). Johnson and colleagues observed that interaction with and acetylation by p300 is not dependent on the B-MYB phosphorylation status since a phosphorylation deficient mutant of B-MYB was readily acetylated in the presence of p300 (Johnson et al., 2002). However, while another group confirmed that this B-MYB mutant binds p300, they also found that the mutant protein was substantially less acetylated. As phosphorylation of B-MYB appears to stimulate its acetylation by p300, this suggests that cyclin A and p300 cooperatively increase the B-MYB transactivation activity (Schubert et al., 2004). In the presence of cyclin D1, acetylation of B-MYB is strongly suppressed as cyclin D1 seems to interfere with the association between p300 and B-MYB (Schubert et al., 2004).

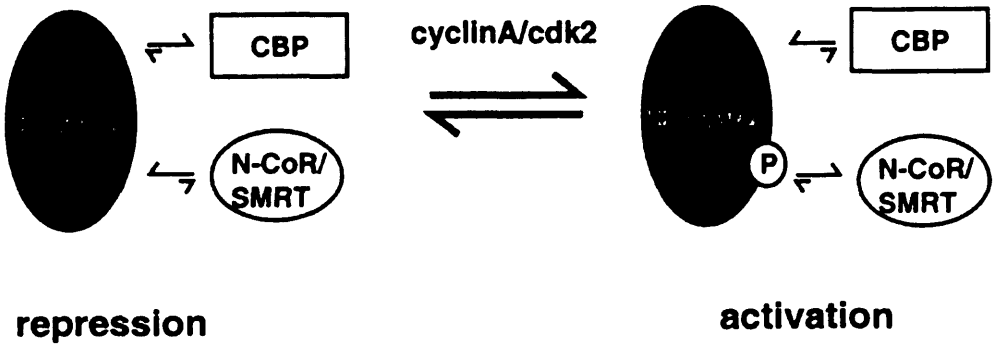


Figure 1-6 Schematic model illustrating the relationship between B-MYB and co-factors

B-MYB interacts with the co-repressors BS69, N-CoR and SMRT, which negatively influence the transactivation potential of B-MYB. Phosphorylation of B-MYB during the S-phase of the cell cycle abrogates the binding of B-MYB to co-repressors and alleviates the cooperation with co-activators such as CBP and p300. The illustration is taken from (Li and McDonnell, 2002).

Taking together these findings, it is conceivable that destruction of cyclin D1 may initiate the disruption of interactions between B-MYB and co-repressors such as N-CoR and SMRT. Subsequently, S-phase dependent phosphorylation of B-MYB would abrogate the remaining interactions with co-repressors and facilitates the association with co-activators such as p300 and CBP rendering B-MYB fully transcriptionally active.

1.2.5.4 Interaction with regulatory proteins

An additional level of control imposed upon B-MYB consists of direct or indirect interactions with a variety of proteins that are responsible for different regulatory functions in the cell. However, while some of these protein interactions affect B-MYB function, they may in turn also influence the activity of the B-MYB binding partners. Therefore, protein interactions affect the capacity of B-MYB to regulate transcription and they allow B-MYB to influence signalling pathways independently of its function as a transcription factor.

An important function of transcription factors is to direct the transcriptional machinery to promoters. Transcription factors can either achieve this directly through interactions with members of the PIC or indirectly through binding to co-factors that bridge the association with the PIC (Lee and Young, 2000). Accordingly, B-MYB cooperates with proteins involved in the process of gene transcription such as TAFII250 (Bartusel and Klempnauer, 2003) and Cdk9 (De Falco et al., 2000).
TAFII250 is a TBP associated factor and it plays a key role in transcription by acting as a scaffold for the assembly of other TAFs and TBP to TFIID. Furthermore, TAFII250 bridges the association between transcription factors and the PIC. Moreover, TAFII250 displays enzymatic activities, including acetyltransferase activity, which facilitate transcriptional initiation (Wassarman and Sauer, 2001). B-MYB requires the presence of TAFII250 to mediate its transactivation ability on MYB binding site (MBS) responsive promoters. Despite the acetyltransferase activity of TAFII250 is necessary for B-MYB transactivation activity no acetylated lysines could be detected in B-MYB in the presence of TAFII250. While the DBD of B-MYB was shown to be required for interaction

with TAFII250 (Figure 1-7), B-MYB and TAFII250 did not associate in a cell free system, suggesting that both proteins interact through a mutual binding partner (Bartusel and Klempnauer, 2003).

Cdk9 is a Cdk1-like kinase and in contrast to other Cdks its kinase activity is constant throughout the cell cycle. Cdk9 associates with T-type cyclins and these complexes phosphorylate the RNAP II C-terminal domain, thus stimulate transcription elongation (Garriga and Grana, 2004). Co-expression of both wild type and a kinase deficient form of Cdk9 decreased the ability of B-MYB to transactivate MBS-dependent and -independent promoters indicating that the inhibitory effect is not dependent on the enzymatic activity of Cdk9. Instead, Cdk9 suppresses the B-MYB transactivation activity through direct interaction with the B-MYB C-terminus (Figure 1-7). Notably, cotransfection of cyclin T1 relives the inhibitory effect of Cdk9 on B-MYB transactivation function (De Falco et al., 2000).

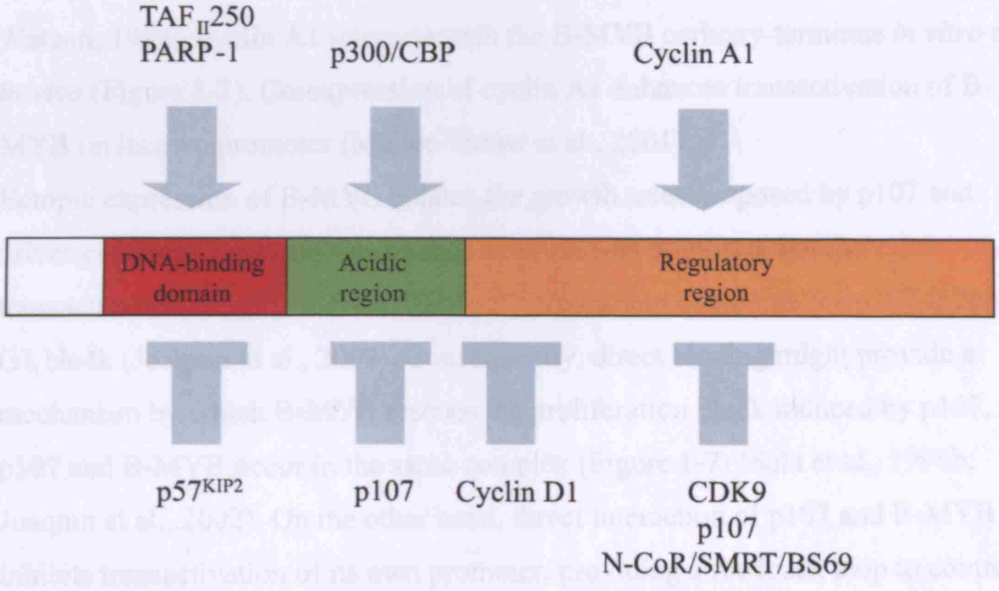


Figure 1-7 Overview of B-MYB regulatory proteins

Different domains of B-MYB have been shown to bind to several B-MYB interacting proteins and the B-MYB domains required for physical interaction are indicated with arrows. These interactions were implicated either in promoting (pointed arrows) or inhibiting (blunt arrows) the transactivation activity of B-MYB.

Another class of B-MYB interacting partners, consists of proteins that are implicated in cell cycle control, such as cyclin D1 (Horstmann et al., 2000b) cyclin A1 (Muller-Tidow et al., 2001), p107 (Sala et al., 1996b) and $p57^{KIP2}$ (Joaquin and Watson, 2003b).

Cyclin D1 strongly inhibits the activity of B-MYB even when a cyclin D1 mutant is used that is unable to bind to Cdks. Because of that, the inhibitory effect of cyclin D1 on B-MYB is thought to be Cdk-independent. Accordingly, B-MYB has been shown to bind to cyclin D1 (Figure 1-7) and the inhibitory effect depends on direct binding of cyclin D1 a central domain between amino acids 265-507 of B-MYB (Horstmann et al., 2000b; Schubert et al., 2004). Considering that cyclin D1 is expressed earlier in the cell cycle than cyclin A, complex formation between cyclin D1 and B-MYB during the G1-phase might enable the accumulation of B-MYB in an inactive form, whereas later in S-phase cyclin A/Cdk2-induced phosphorylation of B-MYB results in its complete activation (Horstmann et al., 2000b).

Cyclin D1 is not the only cyclin that was shown to directly bind to B-MYB. In contrast to cyclin A, which does not occur in a complex with B-MYB (Saville and Watson, 1998), cyclin A1 interacts with the B-MYB carboxy-terminus *in vitro* and *in vivo* (Figure 1-7). Co-expression of cyclin A1 enhances transactivation of B-MYB on its own promoter (Muller-Tidow et al., 2001).

Ectopic expression of B-MYB ablates the growth arrest imposed by p107 and drives cells into S-phase (Sala et al., 1996a). It was proposed that the transactivation activity of B-MYB is not necessary to overcome the p107-induced G1 block (Joaquin et al., 2002). Consequently, direct binding might provide a mechanism by which B-MYB rescues the proliferation block induced by p107, as p107 and B-MYB occur in the same complex (Figure 1-7) (Sala et al., 1996b; Joaquin et al., 2002). On the other hand, direct interaction of p107 and B-MYB inhibits transactivation of its own promoter, providing a feedback loop to control B-MYB autoregulation (Sala et al., 1996b).

Whereas no interaction was detected between the Cdk-inhibitors $p21^{Waf1/Cip1}$ and $p27^{KIP2}$, B-MYB forms a complex with $p57^{KIP2}$ whereby its N-terminal region including the DBD is sufficient for binding to $p57^{KIP2}$ (Figure 1-7). B-MYB was found to partially restore a $p57^{KIP2}$-induced G1 arrest in SAOS-2 cells and $p57^{KIP2}$ suppresses MBS-dependent transcriptional activity of B-MYB through direct physical interaction (Joaquin and Watson, 2003b).

Moreover, B-MYB has been suggested to cooperate with Poly-ADP-ribose polymerase-1 (PARP-1). PARP-1 catalyses the transfer of poly-ADP-ribose units to acceptor proteins and the resulting ADP-ribose polymer interacts with a number of proteins involved in the cellular response to DNA damage and DNA metabolism. Equally, PARP-1 is a molecular sensor of DNA breaks and holds a crucial role in the organisation of their repair. PARP-1 fulfils this function by signalling the presence and the extent of DNA damage so that the cell can establish whether to repair DNA or to undergo apoptosis. A further function of PARP-1 is to support the repair process by increasing accessibility and recruitment of proteins involved in repair to the DNA breaks. Moreover, PARP-1 function has been implicated in transcriptional regulation, telomere cohesion and mitotic spindle formation (Schreiber et al., 2006). PARP-1 binds to the DBD of B-MYB (Figure 1-7) and co-expression of PARP-1 and B-MYB results in synergistic activation of B-MYB responsive promoters (Cervellera and Sala, 2000). The poly-ADP-ribosylation activity of PARP-1 is not required for the synergistic function on MBS-responsive promoters and consistent with this observation no poly-ADP-ribose could be detected on B-MYB (Cervellera and Sala, 2000; Santilli et al., 2001). The physical interaction between PARP-1 and B-MYB is independent of B-MYB phosphorylation, but PARP-1 is unable to exert its co-activator function when B-MYB phosphorylation is inhibited. In fact, PARP-1 has been shown to induce and is required for cyclin A/Cdk2-mediated phosphorylation of B-MYB as cyclin A is unable to enhance the B-MYB transcriptional activity in $PARP^{-/-}$ cells. Thus, it is thought that PARP-1 may facilitate access of the cyclin A/Cdk2 kinase complex to B-MYB and therefore allows its phosphorylation (Santilli et al., 2001).

1.2.6 Upstream pathways regulating B-MYB

As already discussed in the previous sections, B-MYB is a target of mitogen-induced pathways that feed into the cell cycle regulatory machinery. B-MYB is directly regulated by the cyclin D/pRb family/E2F family pathway along with other critical S-phase genes (Lam and Watson, 1993; Zwicker et al., 1996; Liu et al., 1996; Takahashi et al., 2000; Catchpole et al., 2002; Rayman et al., 2002).

Furthermore, B-MYB is negatively regulated by growth suppressors such as p107, p53 and TGF-β (Sala et al., 1996a; Lin et al., 1994; Lam et al., 1992; Satterwhite et al., 1994).

Notably, an increase in B-MYB mRNA expression was detected upon epidermal growth factor (EGF) stimulation (Hanada et al., 2006). EGF signals via interaction with its cognate tyrosine kinase receptor (EGFR), which is frequently overexpressed in human cancers. Extracellular EGF binding induces EGFR dimerisation resulting in autophosphorylation of specific tyrosine residues within the cytoplasmatic portion of the receptor. Various signalling molecules interact with the phospho-tyrosines and this in turn activates a variety of downstream pathways (Yarden, 2001). However, while overexpression of EGFR increased the B-MYB promoter activity, ChIP analysis showed that stimulation with EGF induces binding of EGFR to the B-MYB promoter. This indicates that the EGF induced-activation of the B-MYB promoter occurs independently of downstream targets of EGFR and that EGFR translocates to the nucleus where it contributes to gene expression despite lacking a DBD. Further ChIP assays revealed that binding of nuclear EGFR to the B-MYB promoter occurs in the G1/S phase. Moreover, EGF stimulation induces interaction of EGFR with E2F-1 and their association with the B-MYB promoter, suggesting that E2F-1 and EGFR cooperate in the regulation of B-MYB promoter activity. Mutation of the E2F element in the B-MYB promoter greatly decreased B-MYB promoter responsiveness to EGF stimulation. Taken together these findings, B-MYB is regulated by the EGF pathway and EGFR overexpressing tumour cells might accelerate cellular proliferation by increasing B-MYB expression levels (Hanada et al., 2006).

1.2.7 B-MYB function

1.2.7.1 B-MYB regulates gene expression

The MYB family has been implicated in transcriptional regulation of various target genes involved in the control of cell cycle progression, differentiation and apoptosis (Oh and Reddy, 1999).

All vertebrate MYB proteins are localised to the nucleus and bind a consensus DNA sequence, which is referred to as the MBS. In comparison to other MYB family members, B-MYB differs in that it displays extremely weak or even undetectable transactivation activity in some cell types (Watson et al., 1993; Oh and Reddy, 1999; Joaquin and Watson, 2003b). This probably just reflects the fact, that the B-MYB transactivation ability can be strongly repressed by various means and that B-MYB requires post-translational modifications as well as the interaction with co-activators to be able to exert its transactivation function on some promoters (Lane et al., 1997; Sala et al., 1997; Saville and Watson, 1998; Bessa et al., 2001; Masselink et al., 2001; Li and McDonnell, 2002; Johnson et al., 2002; Schubert et al., 2004). Another factor contributing to the lower transactivation ability of B-MYB might be that the B-MYB DBD binds MBSs with less affinity than c-MYB or A-MYB and is less permissive of binding site variations (Bergholtz et al., 2001).

Despite the fact that all MYB family members are able to recognise the consensus MBS, C/TAACNG (Sala and Watson, 1999), c-MYB and B-MYB display different preferences for nucleotides flanking the core binding site (Mizuguchi et al., 1990; Howe and Watson, 1991). Consequently, the different MYBs transactivate an overlapping set of reporter genes (Oh and Reddy, 1999). As reporter assays are commonly performed with overexpressed proteins, *in vivo* target genes of the different endogenous MYBs might vary considerably due to their individual DNA sequence preferences and their specific interactions with transcriptional co-factors that regulate the local promoter structure. Indeed, individual overexpression of the three MYBs and subsequent microarray assays revealed that each MYB family member regulates a distinctive set of target genes (Rushton et al., 2003).

B-MYB activates the transcription of its target genes in a cell type-specific manner, reflecting its interaction with several co-factors and regulatory proteins. While B-MYB activates numerous genes by direct binding to one or several MBSs, it was also observed that B-MYB activates genes that do not harbour a MBS in their promoters. These findings suggest that, apart from MBS-dependent transactivation, B-MYB also utilises other mechanisms to stimulate transcription. Accordingly, it was proposed that MBS-independent transactivation by B-MYB is mediated though interactions with other transcription factors, whereby B-MYB supports

transactivation as a co-factor (Sala et al., 1999; Cicchillitti et al., 2004; Bartusel et al., 2005).

As expression of B-MYB is induced at the G1/S phase transition, B-MYB might contribute to regulate DNA synthesis through transactivation of target genes involved in the process of DNA replication. For example, B-MYB has been implicated in transcriptional regulation of DNA polymerase α (POLA), which plays a crucial role in the replication of genetic material during the S-phase of the cell cycle. POLA activity is required for primer synthesis to initiate both leading and lagging strand replication (Srivastava and Busbee, 2003). Even though the POLA promoter lacks a MBS, overexpression of B-MYB increased the activity of the POLA promoter fused to a reporter gene (Watson et al., 1993).
Furthermore, B-MYB has been implied in the transactivation of the DNA topoisomerase IIα (TOP2A) promoter (Brandt et al., 1997). TOP2A is indispensable for DNA replication and cell division due to its enzymatic activity that governs cleavage and re-ligation of double-stranded DNA. During replication or gene expression, supercoiled DNA must become accessible and TOP2A contributes in this process by mediating relaxation of DNA supercoils. Moreover, TOP2A has been implicated to play a role in nucleotide excision repair (Kellner et al., 2002). Despite this promoter contains a MBS it has not been investigated whether B-MYB activates the promoter through this site (Brandt et al., 1997).

Transcription of the B-MYB gene and activation of the resulting protein are highly regulated during progression through the cell cycle. According to this intimate linkage with the cell cycle, B-MYB has been implicated in the regulation of genes associated with cell proliferation, particularly of genes involved in the control of G2/M-transition. Thus, B-MYB was proposed to be a factor bridging G1/S control with the expression of G2/M genes (Zhu et al., 2004).
Cyclin B1 and cyclin A are such crucial regulators of G2/M and both have been shown to be B-MYB target genes. It was demonstrated that B-MYB binds to their promoters in ChIP assays and ablation of B-MYB substantially decreases the expression levels of cyclin B1 and cyclin A (Zhu et al., 2004).
Cyclin A1 is another gene that was proposed to be regulated by B-MYB. It was initially believed that B-MYB activates the cyclin A1 promoter through MBSs

(Muller-Tidow et al., 2001). However, a later report revealed that the cyclin A1 promoter is activated through a mechanism involving several Sp1 binding sites as mutation of these elements strongly diminished the effect of B-MYB on the cyclin A1 promoter (Bartusel et al., 2005).
Despite cyclin D1 is an important regulator of the G1 phase, ectopic expression of B-MYB has been shown to increase the level of cyclin D1 (Sala and Calabretta, 1992). Equally, B-MYB-induced activation of the cyclin D1 promoter occurs via Sp1 binding sites (Bartusel et al., 2005).
Ectopic expression of B-MYB increases the level of Cdk1 and, consequently, RNAi-mediated ablation of B-MYB inhibits the expression of Cdk1 (Sala and Calabretta, 1992; Zhu et al., 2004). B-MYB physically interacts with the Cdk1 promoter and mutation of the MBS eliminates binding of B-MYB. Disruption of the MBS also abolishes the activation of the Cdk1 promoter after release of a cell cycle arrest following serum addition (Zhu et al., 2004).

Moreover, B-MYB has been implicated in transcriptional regulation of genes that control various aspects such as proliferation, differentiation, cell survival or the innate immune response.
Notably, B-MYB appears to possess autoregulatory function, as it was initially observed that overexpression of B-MYB resulted in induction of endogenous B-MYB mRNA (Sala et al., 1996b). Despite the B-MYB promoter lacks a MBS, reporter assays have shown that B-MYB can transactivate its own promoter. Subsequently, it was demonstrated that B-MYB and Sp1 synergistically activate the B-MYB promoter through a region, which contains several Sp1 binding sites (Sala et al., 1999). B-MYB physically interacts through its C-terminal domain with Sp1 (Bartusel et al., 2005). Taken together all these findings implies that B-MYB acts as a co-factor of Sp1 (Sala et al., 1999). Furthermore, p107 inhibits B-MYB-induced transactivation of its own promoter. As the two proteins physically interact, it was proposed that p107 might sequester B-MYB and thus prevent it from assisting Sp1 (Sala et al., 1996b).
c-MYC was proposed to be another B-MYB target gene (Nakagoshi et al., 1993; Tashiro et al., 2004). c-MYC is a transcription factor involved in the regulation of proliferation, differentiation and apoptosis; it is a potential oncogene as it immortalises primary cells and is able to transform cells in cooperation with

activated Ras (Fuhrmann et al., 1999). The c-MYC promoter contains several MBSs and is positively regulated by B-MYB in reporter assays (Nakagoshi et al., 1993; Tashiro et al., 2004).

Another B-MYB regulated gene is the insulin-like growth factor-binding protein-5 (IGFBP-5), which belongs to the insulin-like growth factor (IGF) axis. The IGF axis is involved in differentiation and proliferation of embryonic tissues and has been implicated in tumourigenesis. IGFBP family members regulate the activity of IGFs in a tissue-specific manner and IGFBP-5 has been shown to regulate cell survival, differentiation and apoptosis (Beattie et al., 2006). B-MYB can transactivate the IGFBP-5 promoter and overexpression of B-MYB results in increased expression of IGFBP-5 in neuroblastoma cells. The human IGFBP-5 promoter contains two MBSs and B-MYB binds to these sites both *in vitro* and *in vivo* (Tanno et al., 2002).

The fibroblast growth factor-4 (FGF-4) plays a crucial role in different developmental stages, whereby temporally and spatially restricted expression of FGF-4 is essential for proper development (Basilico et al., 1997). Reporter assays in HELA cells showed that B-MYB can greatly stimulate the FGF-4 promoter. The promoter region that confers responsiveness to B-MYB does not harbour a MBS, indicating that B-MYB might activate this promoter through a DNA binding independent mechanism. However, phosphorylation of B-MYB is required for stimulation of the FGF-4 promoter as a phosphorylation deficient mutant is inactive on this reporter construct (Johnson et al., 2002).

The Bcl-2 gene harbours several MBSs, whereas one MBSs was identified just downstream of the P1 promoter and further three MBSs were discovered downstream of the P2 promoter of Bcl-2. *In vitro* binding of B-MYB was demonstrated for the downstream region of both, P1 and P2 (Grassilli et al., 1999; Lang et al., 2005). Interestingly, transactivation of the Bcl-2 promoter through the P1 downstream element was only significantly achieved by B-MYB in conjunction with cyclin A in murine T cells (Grassilli et al., 1999). The P2 downstream element was found to act as an enhancer and B-MYB was able to activate the Bcl-2 promoter consisting of both P1 and P2 elements in a cyclin A-independent manner in human B-cells (Lang et al., 2005). While these reporter assays were performed in different cell lines, it would be interesting to elucidate whether this inconsistency is due to varying cell type-specific levels of cyclin A or whether indeed B-MYB can

mediate transactivation of the Bcl-2 promoter in a cyclin A-dependent as well as in an independent manner. Notably, downregulation of B-MYB with an antisense approach resulted in lower levels of Bcl-2 suggesting that Bcl-2 is a true B-MYB target gene (Lang et al., 2005).

B-MYB also induces transcription of the ApoJ/clusterin promoter (Cervellera et al., 2000). Human cells express a nuclear as well as a secreted isoform of ApoJ/clusterin. Secreted ApoJ/clusterin is a heterodimeric, glycosylated protein found in practically all tissues and all body fluids. Upon cell damage, the other ApoJ/clusterin splice variant is post-translationally modified and translocates to the nucleus. The presence of both the ApoJ/clusterin splice variants has aggravated their functional analysis. Nevertheless, a recent view is that nuclear ApoJ/clusterin acts in a pro-apoptotic and the secreted form in a pro-survival manner, suggesting that tumour cell survival is connected with overexpression of secreted and loss of nuclear ApoJ/clusterin (Shannan et al., 2006). The ApoJ/clusterin promoter harbours a MBS and B-MYB was found to bind to this site both *in vitro* and *in vivo* (Cervellera et al., 2000; Santilli et al., 2005). As a result, mutation of the MBS impairs transactivation of the ApoJ/clusterin promoter by B-MYB. While B-MYB enhances transcription of the ApoJ/clusterin promoter, the activation is greatly increased in combination with cyclin A (Cervellera et al., 2000). Interestingly, retinoic acid induced differentiation of SMCs resulted in downregulation of B-MYB concomitant with downmodulation of only the secreted form of ApoJ/clusterin (Orlandi et al., 2005).

In addition, B-MYB contributes in the control of the heat shock protein 70 (HSP70) (Kamano and Klempnauer, 1997). The expression of HSPs is induced in stress situations whereby they positively modulate cell survival by protecting and disaggregating damaged proteins. During normal conditions, HSPs contribute to protein folding, translocation of *de novo* synthesised proteins and activation of regulatory proteins (Zylicz et al., 2001). B-MYB increases the activity of the human HSP70 promoter and a point mutation in the heat shock element (HSE) of the HSP70 promoter abolished activation following heat shock as well as the ability of B-MYB to transactivate this promoter. A mutant form of B-MYB lacking the DBD was able to transactivate the HSP70 promoter but to a lesser extent than the full-length protein. Therefore, it was proposed that B-MYB is not required to bind directly to DNA in order to activate HSP70. Importantly, expression of C-

terminally truncated B-MYB reduced the transactivation of the HSP70 promoter indicating that the repressed, full-length form of B-MYB that hinders transactivation on many MBS-dependent promoters is able to transactivate HSP70 (Kamano and Klempnauer, 1997).
Moreover, B-MYB has been shown to modulate the innate immune response through the regulation of surfactant protein-A (SP-A), which is a pulmonary host defence protein. SP-A holds a crucial function in the innate host defence by binding and facilitating the uptake of infectious pathogens. Additionally, SP-A binds to the surface of macrophages and contributes in the regulation of their activity (Kingma and Whitsett, 2006). B-MYB activated the SP-A promoter in a cyclin A-dependent manner and mutation of the MBS lowered B-MYB-dependent activity of the SP-A promoter construct. EMSAs have suggested that B-MYB binds directly to this promoter (Bruno et al., 1999).

While B-MYB is generally associated with transcriptional activation, it was shown that B-MYB leads a double life as a transcriptional repressor. The mechanism explaining B-MYB's opposing effect on different promoters is still not clear but it is most likely that it depends on different interactions partners. It appears that the repressor function of B-MYB is restricted to genes that code for ECM components, which are particularly crucial in artery development and atherosclerosis.
During the formation of a developing artery, at first, SMCs proliferate rapidly and then undergo growth arrest. While the expression of ECM components is suppressed in proliferating SMC cells, after growth arrest these cells initiate the synthesis of various ECM components like collagen, elastin and proteoglycans to form the vessel wall. Atherosclerosis on the other hand is elicited as a result of vascular injury, whereby SMCs dedifferentiate, undergo several rounds of proliferation and then synthesise ECM components that form a plaque. Plaque formation can potentially lead to stenosis or rupture of the plaque that may result in thromboembolic complications (Katsuda and Kaji, 2003).
Collagen consists of three polypeptide chains named α chains and a decrease of the promoter activity of the type I α1(I) and α2(I) as well as the type V α2(V) collagen chains was observed upon co-expression of B-MYB in SMCs (Marhamati and Sonenshein, 1996; Kypreos et al., 1999). Interestingly, treatment of SMCs with bFGF causes a significant increase in B-MYB mRNA levels with a concomitant

decrease in the levels of α1(I) collagen mRNA. In accordance, downregulation of B-MYB with antisense oligonucleotides prevented the decrease of α1(I) collagen mRNA levels after bFGF treatment (Kypreos et al., 1998). Furthermore, ectopic expression of B-MYB inhibited the induction of endogenous α1(I) and α2(V) collagen mRNA levels normally observed upon serum deprivation (Marhamati and Sonenshein, 1996; Petrovas et al., 2003). Significantly less α1(I) collagen mRNA was expressed in aortas of transgenic mice that constitutively express B-MYB (Hofmann et al., 2004). Tropoelastin, a soluble monomeric precursor of elastin and soluble elastin levels were also reduced in the aortas of these animals. No apparent differences could be detected in collagen and elastin deposition in wild type versus transgenic animals. However, adult transgenic animals subjected to artery injury showed reduced matrix deposition (Hofmann et al., 2004; Hofmann et al., 2005). In scleroderma fibroblasts, ectopic B-MYB also decreased α1(I) collagen mRNA expression and reporter assays with truncated collagen α1(I) promoter constructs identified a region that was strongly decreased by B-MYB overexpression. EMSAs revealed that B-MYB does not bind to this promoter site but decreases DNA binding of Sp1, which physically interacts with B-MYB. Thus, it was proposed that B-MYB regulates α1(I) collagen expression by interfering with Sp1 (Cicchillitti et al., 2004). The effect of B-MYB on the α2(V) collagen promoter was localised to two elements within exon 1 and was also thought to be mediated via protein-protein interactions (Kypreos et al., 1999).
Notably, cyclin A expression reversed the B-MYB-mediated inhibition and caused an increase in the α2(V) collagen and the elastin promoter activity (Hofmann et al., 2005; Petrovas et al., 2003).

Many of the genes proposed to be targets of B-MYB were studied by reporter assays under conditions where B-MYB was overexpressed and therefore further proof is needed to elucidate whether they are true B-MYB target genes.

1.2.7.2 Cell cycle control

B-MYB is tightly regulated throughout the cell cycle and several genes implicated in cell cycle control are potential B-MYB target genes. While the expression of B-MYB is restricted to proliferating tissues, it is likely that B-MYB function is

important for cell cycle progression. Indeed, a number of studies associate B-MYB with the regulation of cell proliferation, particularly because aberrant expression of B-MYB results in perturbed cell cycle progression in certain cells.

B-MYB downregulation experiments confirmed an involvement of B-MYB in the control of cell cycle progression as B-MYB antisense oligonucleotides inhibited proliferation of different haematopoietic cell lines and glioblastoma cells (Arsura et al., 1992; Lin et al., 1994). Similarly, the number of antibiotic resistant BALB/c 3T3 and LAN-5 clones transfected with B-MYB antisense expression plasmids was significantly lowered (Sala and Calabretta, 1992; Raschella et al., 1995). However, ablation of B-MYB does not appear to affect all cell types in a similar manner, as cell cycle profiles of T98G ganglioblastoma cells with RNAi-mediated ablation of B-MYB showed no marked changes (Zhu et al., 2004). In contrast, by taking a complementary approach, a greater proportion of T98G cells overexpressing B-MYB were found in S-phase compared to control cells after serum stimulation. In asynchronously cycling T98G cells, overexpression of B-MYB also resulted in an increased fraction of cells in S-phase and less cells in G1, although the effect was less prominent in asynchronously growing cells (Sala et al., 1996a). On the other hand, overexpression of B-MYB in U-2 OS or SAOS-2 cells had little effect on the cell cycle profiles, but cotransfection of both B-MYB and cyclin A resulted in a significantly greater portion of S-phase cells (Lane et al., 1997; Sala et al., 1997).

Attempts have been undertaken to address whether B-MYB regulates the progression through a particular cell cycle phase, but contrasting results were obtained. Administration of antisense oligonucleotides against B-MYB inhibited progression from G1- to S-phase of the cell cycle in GM47.23 glioblastoma cells (Lin et al., 1994). Similarly, inhibiting MYB target gene expression with a dominant interfering MYB construct caused ES cells to arrest in G1 (Iwai et al., 2001). Furthermore, B-MYB$^{-/-}$ MEFs showed growth retardation with an increased number of cells in G1 and analysis of BrdU labelled nuclei showed that the staining pattern from B-MYB$^{-/-}$ MEFs was different, indicating that the loss of B-MYB perturbs DNA replication (Garcia et al., 2005).
In contrast to these findings, an arrest in G2/M was observed in *Drosophila* and zebrafish cells lacking a functional B-MYB orthologue (Manak et al., 2002;

Shepard et al., 2005). Reducing B-MYB expression in primary human fibroblasts by RNAi also resulted in a partial block in the G2/M phase of the cell cycle (Santilli et al., 2005). Osterloh and coworkers found that an accumulation of B-MYB depleted fibroblasts in G2/M is due to a delay of the G2 to M phase transition (Osterloh et al., 2007).
The precise temporal requirement for B-MYB during the cell cycle might depend on the cellular systems used for these studies and might vary due to differences in experimental conditions. However, taken together these findings strongly suggest a crucial role for B-MYB to regulate proper cell cycle progression.

While B-MYB affects cellular proliferation, constitutive expression of B-MYB has also been shown to counteract signals that impose a cell cycle arrest. For instance, constitutive expression of B-MYB prevents cell cycle arrest of serum deprived BALB/c 3T3 fibroblasts (Sala and Calabretta, 1992). Nevertheless, overexpression of B-MYB did not prevent growth arrest of serum deprived Swiss 3T3 fibroblasts or T98G glioblastoma cells (Robinson et al., 1996; Sala et al., 1996a). As B-MYB is not sufficient to maintain the latter cell lines in cycle, its proliferation promoting effect presumably depends on the cellular background.
In addition, B-MYB overexpressing glioblastoma cells failed to arrest in G1 upon overexpression of p53 despite the Cdk inhibitor $p21^{Waf1/Cip1}$ was induced. The DBD and to a lesser extent the acidic transactivating domain of B-MYB was shown to be essential for this function and therefore it was proposed that the transcriptional activation of genes downstream of the p53/ $p21^{Waf1/Cip1}$ pathway is involved in bypassing the p53-induced cell cycle arrest (Lin et al., 1994).
Overexpression of either p107 or pRb in SAOS-2 cells results in a decreased number of S-phase cells, but when p107 is expressed in combination with B-MYB a significant increase of cells in S-phase was observed. This effect seems to be specific for p107 as B-MYB had no effect on cells arrested with pRB (Sala et al., 1996a). Whilst deletion of the most N-terminal part of the acidic region (AR) of B-MYB (Δ205-243) was shown to abolish transactivation activity of ectopic B-MYB in U-2 OS cells, this particular B-MYB truncation mutant was able to counteract p107-imposed G1 arrest. This observation suggests that B-MYB-mediated promotion of proliferation does not depend on its ability to transactivate target genes. On the contrary, B-MYB mutants missing other sections of the B-MYB AR

did not efficiently rescue p107-induced cell cycle block and only full-length B-MYB and the B-MYB (Δ205-243) mutant co-immunoprecipitated with p107. While C-terminal truncation of B-MYB enhances its transcriptional activity on several promoters, this truncation mutant failed to bind to p107 and was unable to rescue SAOS-2 cells from p107-induced growth arrest. These findings support the notion that the activation of B-MYB target genes is not required but that the ability of B-MYB to counteract a p107-induced G1 arrest depends on physical interaction between both proteins. Notably, B-MYB and cyclin A formed mutually exclusive complexes with p107 and the Cdk2 kinase activity was enhanced when B-MYB was co-transfected. Consequently, it was proposed that B-MYB might counteract the p107-induced growth arrest by preventing the inhibition of cyclin/Cdk2 kinase activity (Joaquin et al., 2002).
Similarly, ectopic expression of B-MYB could partially restore a $p57^{KIP2}$-induced G1 arrest in SAOS-2 cells. $p57^{KIP2}$-mediated inhibition of cyclin A/Cdk2 kinase activity was partially rescued by co-expresssing B-MYB. As the B-MYB and cyclin A binding sites on $p57^{KIP2}$ overlap and B-MYB competes with cyclin A for binding to $p57^{KIP2}$, a similar mechanism proposed for overriding a p107-induced growth arrest might apply for $p57^{KIP2}$. Hence, B-MYB might promote cell proliferation by a non-transcriptional mechanism that prevents cyclin A/E from binding to $p57^{KIP2}$ (Joaquin and Watson, 2003b).

As discussed in the next section, B-MYB is also capable to override growth inhibiting signals, which would normally lead to terminal differentiation.

1.2.7.3 Differentiation

An important clue that B-MYB might contribute to the regulation of cellular differentiation came form *in situ* hybridisation studies in mouse tissues. B-MYB expression was found to be tightly linked to proliferating tissues and was lost during development when cells underwent terminal differentiation (Sitzmann et al., 1996). Furthermore, a role for B-MYB in differentiation was undermined by the observation that altering B-MYB expression is generally linked to distorted differentiation patterns (Arsura et al., 1992; Raschella et al., 1995; Bies et al., 1996). B-MYB overexpression was found to prevent IL-6-induced growth arrest

associated with terminal differentiation of myeloid leukaemia cells (Bies et al., 1996). B-MYB is downregulated during neuroblastoma differentiation and when overexpressed inhibits neuroblastoma cell differentiation (Raschella et al., 1995; Pagnan and Sala, 2003). Downregulation of B-MYB during neuroblastoma differentiation seems to be primarily controlled at the level of transcription (Raschella et al., 1996). While p130 expression levels gradually raise, pRb and p107 levels increase at early stages of neuroblastoma cell differentiation, followed by a decline at later stages (Raschella et al., 1997). During the course of neuroblastoma differentiation a complex containing p130 bound to the E2F site of the B-MYB promoter in differentiated cells (Raschella et al., 1997). Importantly, transfection of all pRb family members induced neuronal differentiation and downmodulation of the B-MYB promoter activity (Raschella et al., 1998). Therefore, it was proposed that pRb and p107 may contribute to the onset of differentiation, whereas p130 might maintain the differentiated state through transcriptional repression of B-MYB (Raschella et al., 1998). In support of this notion, the B-MYB transcript from mice with a mutated E2F site in the endogenous B-MYB promoter was still produced in senescent cells as well as in the CNS of adult mice supporting the idea that the E2F site is required for silencing B-MYB in senescent and differentiated cells (Tavner et al., 2006).

1.2.7.4 Apoptosis

B-MYB has been shown to modulate apoptosis in different cell types. Primary human fibroblasts require B-MYB for survival as reducing B-MYB expression with siRNA oligonucleotides results in enhanced cell death. Human and murine fibroblasts were also more susceptible to die after thermal injury when they overexpressed the DBD of B-MYB that blocks transcripton of the B-MYB target gene ApoJ/clusterin (Santilli et al., 2005). In contrast, ablation of B-MYB in the chicken lymphoma cell line DT40 does not affect cell survival *per se* and in fact these cells proliferated normally. However, upon DNA-damage cells that lack B-MYB more readily underwent apoptosis (Ahlbory et al., 2005). Constitutive B-MYB expression in IL2-dependent murine lymphocytes resulted in diminished cytokine dependence and awarded the cells resistance to chemotherapeutic agents such as doxorubicin and dexamethasone. Because ectopic B-MYB correlated with

enhanced expression of Bcl-2 it was proposed that B-MYB promotes cell survival through upregulation of Bcl-2 (Grassilli et al., 1999). The proposed anti-apoptotic function of B-MYB in haematopoietic cells was further confirmed with antisense oligonucleotides against B-MYB, which resulted in decreased levels of Bcl-2 and led to increased apoptosis of REH leukaemia cells (Lang et al., 2005). B-MYB overexpressing neuroblastoma cells showed significant resistance to cell death induced by doxorubicin. Furthermore, when the secreted form of ApoJ/clusterin, which is directly regulated by B-MYB, was neutralised with antibodies, doxorubicin-induced apoptosis of neuroblastoma cells was accelerated. Therefore, it was proposed that B-MYB executes its protective effect on neuroblastoma cells through the activation of ApoJ/clusterin (Cervellera et al., 2000).
Interestingly, death evoked by DNA damage or nerve growth factor NGF withdrawal is associated with induction of B-MYB in differentiated neuronal cells (Liu and Greene, 2001). Accordingly, it was observed that derepression of the B-MYB promoter or overexpression of B-MYB induces death of post-mitotic neurons (Liu and Greene, 2001). Antisense and siRNA constructs that block B-MYB expression were shown to provide protection against death (Liu et al., 2004). However, another group challenged these findings as they were not able to observe a change in B-MYB expression levels following DNA damage or NGF withdrawal in differentiated neurons (Pagnan and Sala, 2003). The reason for this controversy is currently not clear, but it will be interesting to elucidate whether B-MYB has indeed an opposing role in transformed compared to post-mitotic neuronal cells.

1.2.8 Is B-MYB a potential oncogene?

In contrast to its c-MYB and A-MYB relatives, no direct involvement of B-MYB has been reported in tumourigenesis so far (Ferrao et al., 1995; DeRocco et al., 1997). This is surprising, considering its diverse functions in various regulatory aspects normally required for transformation. However, while B-MYB displays several characteristics important for the process of transformation, as yet B-MYB has never been shown to contribute to the immortalisation of primary cells.

Despite the lack of unambiguous proof that B-MYB is able to act as an oncogene, B-MYB is highly expressed in many cancer cell lines (Nomura et al., 1988). Furthermore, B-MYB expression has been detected in several primary tumour tissues. For instance, analysis of non-small cell lung cancer biopsies has shown that B-MYB is overexpressed in primary tumours compared with normal tissues (Hibi et al., 1998). B-MYB expression is increased in primary testicular and prostate cancer tissues (Skotheim et al., 2002; Bar-Shira et al., 2002). Moreover, in prostate tumours B-MYB has been linked to metastatic behaviour (Bar-Shira et al., 2002). Similarly, B-MYB expression is upregulated in breast cancers and is associated with poor patient survival (Amatschek et al., 2004). While B-MYB expression is strongly correlated with the proliferative status of a cell, it might be that enhanced B-MYB expression in cancer cells reflects this fact. This suggests that B-MYB expression in transformed tissues might rather be a consequence of transformation than a factor contributing to tumourigenesis. However, the B-MYB locus at 20q13.1 is commonly amplified in breast cancer cell lines (Forozan et al., 2000). While amplification of chromosomal segments may occur as a secondary event during the propagation of cell lines, the B-MYB locus was also found to be amplified in primary human tumours such as in hepatocellular and ovarian carcinomas as well as in cutaneous T-cell lymphomas (Tanner et al., 2000; Zondervan et al., 2000; Mao et al., 2003). Amplification of the B-MYB locus in primary human tumours strongly indicates that B-MYB might be involved in the initiation or progression of human cancer.

Importantly, several lines of evidence gathered in *in vitro* studies, suggest that B-MYB could well possess oncogenic potential. For example, B-MYB overexpressing MEFs escaped activated Ras-induced senescence without inactivation of the $p19^{ARF}$/p53 pathway (Masselink et al., 2001). In addition, ectopic B-MYB results in evasion of a cell cycle arrest imposed by p53 even in the presence of high levels of $p21^{Waf1/Cip1}$ (Lin et al., 1994). These findings suggest that B-MYB functions downstream of p53 and therefore, high B-MYB levels are sufficient to counteract cell cycle arrest mediated by p53.

B-MYB appears to be a crucial member of a pathway that constitutes a central mediator where positive and negative signals act to determine whether the cell goes

on to proliferate or undergoes cell cycle arrest. For instance, transcription of B-MYB is positively regulated by mitogens and by oncogenes like the human papilloma virus oncogene E7, implying that the activity of B-MYB is an important prerequisite for proliferation (Lam et al., 1994). On the other hand B-MYB is negatively controlled by growth suppressors, such as p107, p53 and TGF-β, indicating that downregulation of B-MYB is necessary for the entry into quiescence (Lin et al., 1994; Satterwhite et al., 1994; Sala et al., 1996b).

B-MYB expression has been attributed prognostic power in neuroblastoma tumours. With the use of a semi-quantitative RT-PCR technique, expression of all three MYB family members was assessed in neuroblastoma biopsies. Expression of B-MYB was associated with an increased risk of death independently of N-MYC amplification. Consequently, B-MYB constitutes an important prognostic factor for the remaining neuroblastoma cases that do not display N-MYC amplification (Raschella et al., 1999). Although transcripts of all MYB family members are expressed in primary neuroblastoma biopsies the role of B-MYB is not likely to overlap with that of A-MYB and c-MYB as only B-MYB mRNA expression correlates with patient survival (Raschella et al., 1999).

In apparent contradiction to the findings discussed above, loss of B-MYB has also been associated with genomic instability. For example, lethal mutants of *Drosophila* that do not express DM-MYB show a variety of defects in mitotic cells, including aberrant spindle formation and increased polyploidy and aneuploidy (Manak et al., 2002). Furthermore, a truncation mutant of B-MYB that results in loss of function was identified in a zebrafish genetic screen. Similar to the findings in *Drosophila* cells, mutant zebrafish cells showed defects in spindle formation, which resulted in genomic instability. Moreover, haploid mutant zebrafish displayed an increased cancer incidence after carcinogen treatment, indicating that B-MYB may act as a haploinsufficient tumour suppressor in zebrafish (Shepard et al., 2005). Metaphase spreads of megakaryocytic cells treated with RNAi against B-MYB exhibited different chromosomal abnormalities, indicating that also in higher eukaryotes appropriate B-MYB expression levels are required for the maintenance of genomic integrity (Garcia and Frampton, 2006). These findings may suggest that abnormally low levels of B-MYB could as well contribute to transformation.

A potential role of B-MYB as a tumour suppressor is counter intuitive, but it is possible that B-MYB expression levels need to be carefully balanced in a cell. When B-MYB levels overstep a certain threshold the excess might provide cells of particular tissues with growth advantage that might ultimately facilitate cancer. On the other hand, low B-MYB levels might not be sufficient to properly conduct the various B-MYB functions whereby these cells would loose one of many control mechanisms, rendering them more prone to further cytogenetic abnormalities. However, some cell types or tissues might be more robust towards unbalanced B-MYB levels and sensitivity to aberrant B-MYB levels might depend on factors such as the developmental stage.

In summary, while B-MYB has never been shown to immortalise primary cells, it is able to rescue growth arrests imposed by various means, render cells growth factor independent, inhibit differentiation and act as a pro-survival factor under certain circumstances. Moreover, B-MYB synergistically cooperates with activated Ras in the transformation of primary cells and its expression levels were found to be elevated in various human malignancies. All these characteristics are in accordance with a gain of function as it is defined for a proto-oncogene. Taken together these findings strongly suggest that B-MYB plays an important role in maintaining cellular homeostasis and that deregulated expression of B-MYB could play an important role in transformation by providing conditions favourable for the accumulation of further genetic abnormalities.

1.3 Neuroblastoma

Neuroblastoma is a malignant, embryonic cancer deriving from primitive cells of the sympathetic nervous system, which is formed by the sympathetic side chains, the sympathetic ganglia and the adrenal medulla (Maris and Matthay, 1999; van Noesel and Versteeg, 2004). The sympathetic nervous system is part of the autonomic nervous system and regulates involuntary body functions in response to stress signals. The chromaffin cells of the adrenal medulla produce the catecholamines adrenaline and noradrenaline, which the body requires to perform optimally in response to stress situations (Sadler, 1985). Progenitor cells of the sympathetic nervous system arise from the neural crest. These precursor cells generally maintain gene expression profiles and the differentiation capacity reminiscent of their embryonic origin (Nakagawara and Ohira, 2004).

The clinical behaviour of neuroblastoma varies widely, ranging from dissemination with fatal outcome to spontaneous regression that frequently occurs in patients with localised disease. The incidence of spontaneous regression in neuroblastoma is up to 100 times greater than that for any other human cancer (Pritchard and Hickman, 1994). Particularly in infants, tumours can mature into ganglioneuromas, which are a sub-category of differentiated benign tumours consisting of clusters of mature neurons surrounded by stromal Schwann cells. In contrast, highly malignant neuroblastomas are undifferentiated tumours of small, round neuroblasts (van Noesel and Versteeg, 2004). Older patients often die from a very aggressive, metastatic disease despite intensive treatment and about 40% of all cases at diagnosis are high risk tumours (Matthay, 1995; Westermann and Schwab, 2002).

Neuroblastoma accounts for 8-10% of all paediatric cancers with an annual incidence of about 1 in 100,000 children under the age of 15 years, which makes it the most common extracranical solid tumour in children (Sawada et al., 1987; Bernstein et al., 1992). The median age at diagnosis is about 18 month. 60% of all neuroblastoma cases occur in children under two years of age and almost all cases are diagnosed in children up to ten years. (Matthay, 1995).

1.3.1 Neural crest cells

The neural crest is a proliferative, migratory and multipotent progenitor cell population that only occurs in vertebrate embryos (Kalcheim and Burstyn-Cohen, 2005). During human embryogenesis, the nervous system forms from the ectoderm. At the beginning of the third week, a structure known as the neural plate appears in the dorsal region of the embryo. Subsequently the edges of the neural plate become elevated until they finally fuse and form the neural tube. Neural crest cells arise from the ectoderm at the edges of the neural plate. Following neural tube closure, these cells delaminate from the dorsal part of the neural tube, and migrate extensively to populate distant sites throughout the embryo along specific pathways (Figure 1-8) (Dorsky et al., 2000; Tucker, 2004). Neural crest cells are capable of differentiating into diverse cell types producing most of the neurons and glia of the peripheral nervous system, melanocytes and nearly all of the elements of the craniofacial skeleton (Sadler, 1985; Kalcheim and Burstyn-Cohen, 2005).

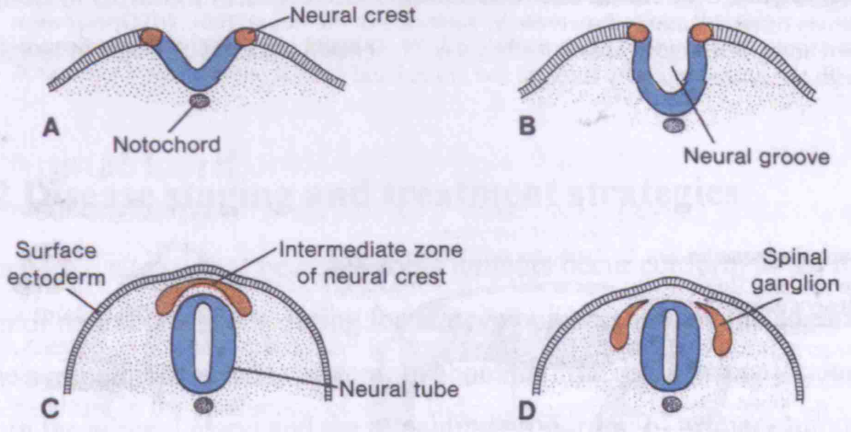


Figure 1-8 Migration of the neural crest cells

The neural crest cells constitute an early cell population, which forms at the dorsal part of the neural folds. Following the closure of the neural folds, the neural crest cells leave the dorsal aspect of the neural tube to form the neurons and glia cells of the peripheral nervous system. Illustration from (Sadler, 1985).

Initially, neural crest cells invade the dorsolateral part of the neural tube and form the sensory and dorsal root ganglia. Later on in development, neural crest cells of the thoracic region migrate behind the dorsal aorta to form chains of sympathetic ganglia on each side. Some of the sympathetic neuroblasts migrate in front of the aorta to differentiate into the preaortic ganglia and others migrate to the heart, lungs and gastrointestinal tract to form the organ plexuses. A portion of the sympathetic neuroblasts also migrates to the developing adrenal to form the medulla (Sadler, 1985).

While neural crest cells are generally believed to be multipotent, it was proposed that only a proportion of these cells retains the capability to differentiate into distinct lineages and clonal analysis showed that not all neural crest cells can develop into any specific lineage. Therefore, premigratory neural crest cells are a heterogeneous cell population containing cells with a clearly determined fate as well as cells able to acquire the characteristics of the different lineages. Extracellular signals such as Wnts, BMP2/BMP4 and TGFβ1,2,3 prompt the formation of different lineages and contribute to the diversity of target tissues (Dorsky et al., 2000; Tucker, 2004).

1.3.2 Disease staging and treatment strategies

The anatomic sites where neuroblastoma tumours occur conform to the migration pattern of neural crest cells during foetal development, namely the adrenal medulla and the sympathetic nervous system. In about half the cases primary tumours are found in the adrenal gland and the remaining proportion of primary tumours occurs at paraspinal sympathetic ganglia in the chest, abdomen or pelvis. Neuroblastoma tumours give rise to common clinical symptoms including abdominal pain, weight loss, respiratory difficulties and fever (Matthay, 1995). Metastases occur in almost 70% of patients and the most common sites are the bone marrow, bone, liver and lymph nodes. Secondary metastases were reported to spread to the lungs and the brain (Matthay, 1995).

Current prognostic evaluation is based predominantly on the degree of tumour spread and the patient age. For reasons unknown at present, patient age at diagnosis strongly influences the clinical prognosis. Generally, infants diagnosed before year 1 are curable with surgery and little or no adjuvant therapy. Conversely, neuroblastoma in older children frequently shows metastases at diagnosis and most of these patients die from disease progression despite intensive chemotherapy (van Noesel and Versteeg, 2004).

Six different stages describing neuroblastoma progression are distinguished and staging is commonly performed according to the International Neuroblastoma Staging System (INSS) (Table 1-1).

Table 1-1 INSS

Stage	Description
1	Localised tumour confined to the area of origin; complete gross excision, with or without microscopic residual disease; identifiable ipsilateral and contralateral lymph nodes negative microscopically
2A	Unilateral tumour with incomplete gross excision; identifiable ipsilateral nonadherent lymph nodes negative microscopically
2B	Unilateral tumour with complete or incomplete gross excision; with positive ipsilateral nonadherent lymph nodes; identifiable contralateral lymph nodes negative microscopically
3	Tumour infiltrating across the midline with or without regional lymph node involvement; or unilateral tumour with contralateral regional lymph node involvement; or midline tumour with bilateral regional lymph node involvement or extension by infiltration
4	Dissemination of tumour to distant lymph nodes, bone, bone marrow, liver or other organs
4S	Localised primary tumour as defined for stage 1 or 2 with dissemination limited to liver, skin and/or bone marrow (<10% tumour) in infants younger than 1 year

Adapted from (Matthay, 1995).

Localised tumours are divided into stages 1 and 2, based on regional lymph node status. Stage 3 indicates unresectable tumours, which have spread across the midline and stage 4 tumours show distant metastases and bone marrow involvement generally in patients older than 1 year (Papaioannou and McHugh, 2005). Generally, patients diagnosed with the disease stages 1 and 2 have a good prognosis whereas stage 3 and 4 indicate a poor prognosis. More recently, stage 4S was included in the staging system. Although 4S tumours present like a metastatic disease, they do not show negative prognostic factors such as N-MYC amplification as well as large bone marrow involvement and children with these tumours have a high survival rate (Papaioannou and McHugh, 2005).

Apart from the clinical pattern of disease spread and age at diagnosis, it is widely believed that tumour histological assessments describing the degree of differentiation and the extent of stroma have prognostic value (Shimada et al., 1999). Furthermore, specific genetic and molecular features such as chromosome number (ploidy), allelic loss, oncogene amplification and abnormal gene expression have been correlated with clinical outcome. Only more recently, several of these biological markers have been incorporated in the prognostic evaluation. Accordingly, the INSS allows clinical sub-groups to be identified upon which the above mentioned risk factors can be determined. Risk stratification based on clinical, genetic and molecular features allows the best possible diagnosis, prognosis and therapy decisions to date. The main characteristics that define the different neuroblastoma risk groups are summarised in Table 1-2.

Survival rates after treatment range from 95% in stage 1 to 25% in stage 4 patients with unfavourable features (Henry et al., 2005). Low risk neuroblastoma patients are commonly treated with surgery alone, whereby stage 1 and 2A patients have a 5 year event free survival rate greater than 90%. While stage 1 and 2 patients normally have high survival rates, they are considered at high risk when N-MYC is amplified or when they display unfavourable histology. Stage 4S patients have a 5 year event free survival of 70-90% and exhibit a high frequency of spontaneous regression. Stage 3 and 4 patients without amplified N-MYC are categorised as intermediate risk group, which has a 5 year event free survival of 60-80%. The high

risk group consists of stage 3 and 4 tumours of older patients with N-MYC amplification. Treatment of high risk patients includes intensive chemotherapy, surgery and in some cases radiation therapy. Regardless of intensive treatment regimens, high risk patients only have a 5 year event free survival of 25-30% (van Noesel 1997).

Table 1-2 Characteristics of neuroblastoma risk groups

Tumour characteristics	Low risk	Intermediate risk	High risk
Clinical:			
Age	< 1 year	> 1 year	> 1 year
Stage	1, 2, 4s	3, 4	3, 4
5-year survival	95%	50%	25%
Genetic:			
Ploidy	3N	2N/4N	2N/4N
1p loss	Rare	Rare	Frequent
11q loss	Rare	Frequent	Rare
14q loss	Rare	Frequent	Rare
17q gain	Rare	Frequent	Frequent
N-MYC	Normal	Normal	Amplified
Molecular:			
TrkA	High	Low	Low
TrkB	Low	Low	High
TrkC	High	Low	Low
$p75^{NTR}$	High	?	Low

Adapted from (van Noesel and Versteeg, 2004).

Despite enduring aggressive treatment regimens, the low survival probability of high risk patients demands further research into improved therapeutic procedures. Current research aims to find alternative approaches to support or substitute common treatment strategies. Among them, application of retinoids has shown to

improve survival of high risk patients (Matthay et al., 1999; Reynolds et al., 2003).
The vitamin A metabolite retinoic acid (RA) is important for the nervous system development. RA serves as a regulator for gene expression by binding to RA receptors, which directly modulate target gene expression. One way by which RA regulates neurogenesis is through transcriptional control of neurotrophin receptor genes (Clagett-Dame et al., 2006). Application of 13-cis retinoic acid after bone marrow transplantation has become standard practice in the management of high risk neuroblastoma patients (Brodeur, 2003; Reynolds et al., 2003).
Another strategy in the fight against neuroblastoma is induction of apoptosis. Fenretinide, a synthetic retinoid, induces apoptosis instead of differentiation of neuroblastoma cells. This agent is currently undergoing clinical trials (Reynolds et al., 2003).
Targeted radiotherapy aims at treating the disease with minimal damage to other tissues and such an approach involves the use of radioactively labelled Meta-iodobenzyl-guanidine (MIBG). This compound is structurally related to noradrenaline and is actively taken up by neuroblastoma cells. Importantly, MIBG has been shown to effectively treat advanced stage neuroblastoma patients (de Kraker et al., 1995; Yanik et al., 2002).
Furthermore several other approaches, including the use of antibodies against tumour surface markers or specific delivery of antisense compounds were promising in mouse models and await clinical trials in neuroblastoma patients (Raffaghello et al., 2003; Brignole et al., 2004).

1.3.3 Genetic abnormalities occurring in neuroblastoma

Neuroblastoma is a particularly heterogenic disease and the causes that lead to neuroblastoma tumour initiation and progression are still largely unclear (Maris and Matthay, 1999). However, multiple somatic defects, such as alterations in cell ploidy and gain or loss of alleles have been identified in various neuroblastoma biopsies. Such non-random genetic alterations associated with neuroblastoma are presented in the following sections. While some of these changes are currently included in the diagnosis decision, others await proof of their relevance in this disease. Incorporation of additional prognostic markers might allow a more

accurate stratification of tumours at presentation and could help to better identify more appropriate therapeutic strategies for each individual patient.

1.3.3.1 Chromosomal abnormalities

1.3.3.1.1 Ploidy

Aneuploidy, which describes the loss or gain of chromosomes, is frequent in neuroblastoma and the DNA content of neuroblastoma cells is significantly associated with disease outcome (Westermann and Schwab, 2002). Cytogenetic analyses have allowed the identification of 4 ploidy levels, according to which neuroblastoma tumour cells may be near-diploid, near-triploid, near-tetraploid or even near-pentaploid. Near-diploid and near-tetraploid tumours mostly occur in patients over 1 year of age whose neuroblastoma cells harbour chromosomal abnormalities including N-MYC amplification and deletion of chromosome 1. Such genetic alterations correlate with advanced stages of the disease, with rapid tumour progression and poor prognosis. Accordingly, the majority of high risk patients have near-diploid or near-tetraploid tumours (Kaneko et al., 1987; Hayashi et al., 1989; Kaneko et al., 1990). The near-triploid tumours are characterised by three almost complete haploid sets of chromosomes with only few chromosomal abnormalities. Near-triploid tumours are predominantly detected in patients younger than 1 year, including INSS stages 1, 2 and 4s, with positive clinical outcome. Equally, near-pentaploid tumours indicate a favourable prognosis and prolonged disease free survival (Kaneko et al., 1987; Hayashi et al., 1989; Kaneko et al., 1990).

The mechanism underlying the formation of aneuploid neuroblastoma cells still remains elusive. However, it has been proposed that aneuploidy might arise from tetraploidisation followed by bipolar, tripolar or tetrapolar cell divisions (Kaneko and Knudson, 2000).

1.3.3.1.2 Amplification of 17q

Gain of a segment of the long arm of chromosome 17 (17q) occurs in more than 80% of all neuroblastoma cases, thus making it the most frequent genetic

abnormality in this disease (van Noesel and Versteeg, 2004). 17q amplification has been observed to result from either a gain of 17q in an unbalanced translocation or as part of a whole chromosome gain. Unbalanced translocations, whereby a segment on a partner chromosome is lost and replaced by a fragment of 17q most often occur with chromosome 1p (Caron et al., 1994; Savelyeva et al., 1994). While amplification of the whole chromosome 17 is associated with favourable outcome, selective gain of 17q correlates with more aggressive neuroblastomas, patient age of older than one year, N-MYC amplification and 1p loss. Multivariate analyses comprising factors such as age, tumour stage and N-MYC amplification revealed that including the status of 17q significantly increases the predictive power (Bown et al., 1999).

Even though the 17q breakpoints are heterogeneous, a commonly amplified region has been determined. Consequently, it was suggested that this locus harbours a yet to be identified oncogene (Maris and Matthay, 1999; Brodeur, 2003). The most important oncogenic candidates located on the commonly translocated 17q segment are survivin and NM23-H1.
Survivin is an inhibitor of apoptosis and acts by negatively regulating caspase-9. The gene encoding for survivin is located to 17q25, which is within the minimal region of 17q chromosomal gain. Increased expression of survivin was found to correlate with unfavourable histology and adverse clinical factors (Islam et al., 2000). Interestingly, overexpression of survivin in a neuroblastoma cell line leads to inhibition of retinoic acid-induced apoptosis, a finding, which might explain its functional role in this disease (Islam et al., 2000).
NM23-H1 localises to 17q22, a chromosomal region, which is also within the minimal region of 17q chromosomal gain. NM23-H1 is observed in aggressive neuroblastomas and when overexpressed it was shown to promote metastasis of neuroblastoma cells (Almgren et al., 2004).

1.3.3.1.3 Deletion of 1p

Deletion of the short arm of chromosome 1 (1p) occurs in 30-35% of all neuroblastoma tumours (van Noesel and Versteeg, 2004). The smallest region of overlapping deletion occurs within 1p36.3, but so far there has been no agreement

about the proximal and distal break points (Bauer et al., 2001; van Noesel and Versteeg, 2004). Whereas in neuroblastoma tumours with amplification of N-MYC loss of heterozygosity of 1p usually affects large areas, the deleted region was found to be smaller in tumours with N-MYC single copy numbers. Consequently, tumours with a smaller deleted region in 1p are often near-triploid and associate with a better outcome (van Noesel and Versteeg, 2004).
1p deletion occurs more commonly in children with advanced stages of neuroblastoma. In high risk patients it is strongly associated with structural abnormalities such as N-MYC amplification and gain of chromosome 17q (Matthay, 1995; Brodeur et al., 1997). However, deletion of 1p is considered an independent prognostic factor of unfavourable outcome in neuroblastomas of every stage (Maris et al., 2000; Mora et al., 2000).

Loss of 1p is frequently associated with an unbalanced translocation involving 17q. However, as yet no genes have been identified at the break-points that could contribute to the generation of a fusion gene. This might be due to the fact that the breakpoints are highly variable on both chromosomes (van Noesel and Versteeg, 2004).

Re-addition of portions of chromosome 1p to a 1p-deleted neuroblastoma cell line induced differentiation or cell death (Bader et al., 1991). This suggests that one or even several still unidentified tumour suppressor genes are located within this region. Recently, the chromodomain helicase DNA binding domain 5 (CHD5) has been identified as a 1p tumour suppressor gene (Bagchi et al., 2007) and it will be interesting to assess its significance in neuroblastoma.

1.3.3.1.4 Deletion of 11q

Various genetic alterations were observed involving the long arm of chromosome 11 (11q). These aberrations include balanced translocations involving 11q21 and 11q22, deletion of 11q23-ter, inversion of 11q21-q23 and more frequently allelic loss (Westermann and Schwab, 2002). 15-20% of neuroblastoma tumours show chromosome 11q deletions (Mertens et al., 1997). 11q deletions are predominantly

large and terminal and a single region within 11q23.3 was deleted in all tumours with 11q loss of heterozygosity (Guo et al., 1999).

Loss of 11q was shown to have a strong inverse correlation with N-MYC amplification. 11q deletions frequently correlate with stage 4 neuroblastoma tumours without N-MYC amplification and therefore define a distinct sub-set of stage 4 patients. This sub-group of patients also shows gain of 17q, losses of 3p, 4p and 14q as well as an inverse correlation with 1p deletion (Vandesompele et al., 2001; Plantaz et al., 2001).

It is commonly believed that a yet to be identified tumour suppressor gene might be located at 11q as the transfer of chromosome 11 into a neuroblastoma cell line resulted in cellular differentiation (Bader et al., 1991).

1.3.3.1.5 Other chromosomal alterations

Additional recurrent DNA sequence copy number changes involving amplification of 1q as well as deletion of 2p, 3p, 9p and 14q were identified with variable frequency in neuroblastoma tumours.

Chromosomal gain at 1q21-q25 was found in 50% of stage 4 neuroblastoma tumours. Moreover, survival analysis indicated that 1q21-q25 gain was associated with a poor disease outcome (Hirai et al., 1999).

Deletion of 2q33 has been linked with loss of expression of caspase-8, encoded on this locus (Teitz et al., 2000; Takita et al., 2001). Neuroblastoma cell lines that do not express caspase-8 were resistant to death receptor- and doxorubicin-mediated apoptosis. Importantly, induction of apoptosis was restored upon ectopic expression of caspase-8, suggesting that this protein may act as a tumour suppressor (Takita et al., 2001). Apart from gene deletion, the caspase-8 gene was also found to be silenced through DNA methylation in neuroblastoma cell lines (Teitz et al., 2000; Takita et al., 2001).

Loss of 3p frequently occurs in neuroblastoma and is associated with adverse clinical outcome. This deletion appears to be inversely correlated with N-MYC amplification and thus defines a high risk subgroup in patients with N-MYC single copy (Spitz et al., 2003).

Loss of 9p21 occurs frequently in neuroblastomas detected by mass screening (Westermann and Schwab, 2002). The tumour suppressor gene CDKN2A, that encodes both $p16^{INK4A}$ and $p14^{ARF}$ maps to 9p21. Homozygous deletion of CDKN2A has been detected in 4 out of 46 neuroblastoma cell lines (Thompson et al., 2001).

Another common cytogenetic abnormality in neuroblastoma is loss of 14q. The smallest common region of allelic loss is a region in 14q32 of approximate 1.1 Mb. Interestingly, loss of 14q associates with loss of 11q in N-MYC single copy tumours and it occurs almost exclusively in patients with advanced stages of disease (Hoshi et al., 2000).

In summary, neuroblastoma is a genetically complex disease with a number of recurrent chromosomal abnormalities that result from loss or gain of genetic material. While some chromosomal aberrations are relevant in terms of diagnosis and prognosis, the precise mechanisms by which they lead to the development and progression of neuroblastoma are still not fully understood.

1.3.3.2 Altered gene expression

Apart from gains or losses of whole chromosome regions, activation or suppression of particular genes appears to be another strategy of neuroblastoma tumour cells to obtain growth advantage, as aberrant gene expression might distort the carefully balanced cellular regulation circuits. Neuroblastoma displays several such alterations in gene expression, which are discussed in the following sections.

1.3.3.2.1 N-MYC amplification

Amplification of N-MYC is one of the most prominent abnormalities found in a subset of neuroblastomas. The proto-oncogene N-MYC is a transcription factor that is important in cellular proliferation and is expressed in the developing nervous system as well as in a subset of other tissues (Wenzel and Schwab, 1995).

N-MYC amplification occurs in about 22% of primary neuroblastomas but accounts for 50% of tumours classified as stage 3 and 4 (Brodeur et al., 1997). While as yet no mutations have been detected in the N-MYC coding sequence from neuroblastoma tumours, N-MYC amplification results in high levels of N-MYC mRNA and protein expression (Schwab et al., 1984). Taken together these findings strongly imply that the effect of N-MYC amplification in neuroblastoma tumours results from increased oncogenic dosage, which contributes to tumourigenesis.

N-MYC is located on chromosome 2p23-24 and *in situ* hybridisation analyses revealed that when N-MYC is amplified it occurs in cell lines predominantly as homogeneously staining regions (HSRs) on different chromosomes. In contrast, in primary tumours amplified N-MYC is mainly located in double-minute chromosome bodies (DMs), which contain 50-500 copies of the N-MYC gene (Brodeur, 2003). DMs are extrachromosomal circular stretches of an amplified DNA region and when the amplified DNA region is integrated into a chromosomal locus a HSR is generated. Although N-MYC is frequently amplified, both parental copies are usually retained at the original locus (Corvi et al., 1994). Interestingly, N-MYC copy number is usually consistent within a tumour and at different times during disease progression (Brodeur et al., 1987). The amplified DNA harbouring N-MYC can range from 100 kilo bases to more than 1 Mega bases, but a core sequence encompassing N-MYC has been found consistently without rearrangements. While the large size of amplified DNA harbours additional genes, N-MYC has emerged as the only consistently amplified gene (Westermann and Schwab, 2002).

N-MYC amplification is highly predictive for the clinical outcome whereby it serves as an indicator of rapid tumour progression and indicates poor prognosis in

all stages of the disease (Brodeur et al., 1984; Seeger et al., 1985; Maris and Matthay, 1999). Patients with tumours that lack N-MYC amplification have a 5 year event free survival of 60%, whereas only 10% of patients with highly amplified N-MYC survive for a 1-year period indicating a strong correlation between N-MYC amplification and tumour aggressiveness (Seeger et al., 1985). Notably, there is a close association between amplified N-MYC and 1p deletion. Whilst most tumours with N-MYC amplification show loss of 1p as well, N-MYC is not amplified in all cases with 1p deletion (Brodeur et al., 1997). Furthermore, N-MYC amplified tumours are strongly associated with additional structural abnormalities like gain of 17q, near-diploidy or near-tetraploidy (van Noesel and Versteeg, 2004).

The significance of N-MYC amplification has been addressed in various experimental systems. For example, overexpression of N-MYC induces morphological transformation, anchorage-independent growth, and tumourigenicity in embryonic rat cell cultures (Small et al., 1987). Ectopic expression of N-MYC in neuroblastoma cells and post-mitotic sympathetic neurons promotes S-phase entry (Lutz et al., 1996; Wartiovaara et al., 2002). Moreover, following DNA damage N-MYC overexpression in neuroblastoma cells has been shown to cause aberrant amplification of the centrosomes, which is associated with the formation of multipolar mitotic spindles and imbalanced segregation of chromosomes (Sugihara et al., 2004). A direct role for N-MYC in the generation of neuroblastoma has been demonstrated by overexpressing N-MYC in an *in vivo* model. Expression of N-MYC was directed to cells derived from the neural crest and several N-MYC transgenic mice developed tumours. These tumours arose in a dose dependent manner and occurred in the thorax and abdomen, whereby the tumour histology was consistent with that of human neuroblastoma (Weiss et al., 1997). This study proves that amplification of N-MYC induces tumourigenesis *in vivo* and presents a valuable model to study different aspects of neuroblastoma.

Mechanistically, N-MYC might contribute to cellular transformation through the regulation of target genes. For instance, it was proposed that N-MYC is able to promote cell growth by activating genes involved in protein synthesis and ribosome biogenesis (Boon et al., 2001). Microarray analysis of RNA from primary

neuroblastoma cell cultures showed a higher expression of MCM7 in N-MYC amplified versus non-amplified tumours. MCM7 is required for DNA replication and thus direct activation of MCM7 might contribute to neoplastic transformation of these N-MYC-amplified tumours (Shohet et al., 2002). Moreover, ChIP analyses identified the p53 ubiquitin ligase MDM2 as an N-MYC target gene (Slack et al., 2005).

1.3.3.2.2 Neurotrophin receptors

Malignant transformation leading to neuroblastoma may be due to delayed or absent induction of neuronal differentiation. Although the transformation event occurring in neuroblasts is not understood in detail, it is thought that different neurotrophin receptors are involved.

Neurotrophins, the high affinity ligands of the neurotrophin receptors, are soluble, secreted glycoproteins that regulate growth, development, survival and repair of the nervous system. The neurotrophin family include nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 and neurotrophin-4 (NT-3 and NT-4). During development, neuronal survival is absolutely dependent on constant exposure to optimal amounts of neurotrophins. Neurotrophins use two classes of signalling transmembrane receptors, the Trk tyrosine kinase receptors and the p75 neurotrophin receptor (p75NTR), which are believed to play a crucial role in the development of neuroectodermal tumours by controlling the differentiation and survival of neuroblasts. These receptors can either support or inhibit each other and the development and survival of neurons therefore depends on the functional interplay of the downstream signalling cascades generated by Trk and p75NTR receptors (Kaplan and Miller, 2000; Patapoutian and Reichardt, 2001; Hempstead, 2002). The expression of neurotrophin receptors on the cell surface is a distinct feature of neuroblastoma tumours and differential expression of neurotrophin receptors has prognostic value (Maris and Matthay, 1999; van Noesel and Versteeg, 2004).

The Trk receptor family is composed of TrkA, TrkB and TrkC and their expression is regulated during development. NGF activates TrkA and in the presence of NGF, developing neurons differentiate, but in the absence of this ligand they undergo apoptosis (Kaplan and Miller, 2000). TrkB serves as receptor for BDNF as well as

NT-4 and TrkC binds NT-3. In addition, it was shown that NT-3 can also activate TrkA and TrkB (Patapoutian and Reichardt, 2001).
p75NTR binds to all neurotrophins and acts as a co-receptor for Trk receptors, whereby it selectively modulates the Trk-mediated response of neurons to neurotrophins (Clagett-Dame et al., 2006). For instance, the TrkA and the p75NTR receptors collaborate to generate high-affinity binding sites for NGF. Additionally, the p75NTR receptor negatively regulates the binding of NT-3 to activate TrkA and TrkB (Patapoutian and Reichardt, 2001). p75NTR can interact directly with Trk receptors and its signalling capacity is modified by the concomitant activation of Trk receptors (Kaplan and Miller, 2000). In contrast to the Trk receptors, p75NTR transmits both pro-survival and pro-apoptotic signals. It is believed that p75NTR only mediates apoptosis when Trk receptors are inactive or sub-optimally activated (Kaplan and Miller, 2000).

The expression patterns of the different neurotrophin receptors correlate with the biological and clinical features of neuroblastoma (Maris and Matthay, 1999). For example, TrkA expression is a favourable prognostic marker and the absence of TrkA mRNA expression predicts poor patient survival (Nakagawara et al., 1993; Suzuki et al., 1993). This might be explained by the fact that abundant levels of TrkA occur in mature sympathetic ganglia (van Noesel and Versteeg, 2004). The expression of TrkA correlates with younger patient age, lower disease stage and absence of N-MYC amplification (Nakagawara et al., 1993; Suzuki et al., 1993). Accordingly, tumours that lack TrkA mRNA expression display a low fraction of Schwann cells and the tumour cells are generally undifferentiated (Nakagawara et al., 1993; Suzuki et al., 1993). Interestingly, ectopic expression of TrkA in neuroblastoma cell lines induced differentiation in the presence of NGF (Lavenius et al., 1995). A TrkA splice variant, named TrkAIII has been described whose expression is restricted to undifferentiated neural crest progenitors. This NGF-unresponsive isoform was found more predominantly in advanced stages of neuroblastoma and is oncogenic in NIH3T3 cells (Tacconelli et al., 2004).
TrkB is preferentially expressed in aggressive neuroblastomas and correlates with N-MYC amplification (Nakagawara et al., 1994). It was observed that TrkB expression is often accompanied by overexpression of its ligand BDNF. This receptor-ligand expression represents an autocrine survival pathway that seems to

contribute to enhanced drug resistance in tumours (Ho et al., 2002). In contrast, Trk C expression is predominantly found in lower stage, N-MYC single copy tumours (Yamashiro et al., 1997).
Interestingly, it was proposed that TrkB and TrkC are expressed early in the development of sympathetic ganglions whereas a switch to TrkA expression occurs in mature ganglion cells. Hence, the expression pattern of Trk receptors may reflect the level of differentiation at the time of transformation of the neuroblast (van Noesel and Versteeg, 2004).
The prognostic significance of p75NTR, independent of Trk expression, in neuroblastoma is still unclear but high p75NTR expression levels occur preferentially in lower risk neuroblastomas and N-MYC amplification correlates with low expression of p75NTR (Kogner et al., 1993).

1.3.3.2.3 Other abnormal patterns of gene expression

While p53 is frequently mutated in different human cancers, p53 sequence alterations are rarely observed in neuroblastoma tumours (Vogan et al., 1993). However, in neuroblastoma other mechanisms might apply that result in p53 inactivation. For instance, it was observed that 96% of undifferentiated neuroblastomas showed elevated levels of wild-type p53 in the cytoplasm and lacked nuclear p53 staining. Therefore, it was proposed that p53 may be sequestered in the cytoplasm in undifferentiated neuroblastomas, thus render it functionally inactive (Moll et al., 1995).

p73 is a homologue of p53 and it induces apoptosis similar to p53, especially during neuronal development (Jacobs et al., 2004). However, unlike p53-deficient mice, p73 knock out animals do not develop spontaneous tumours (Melino et al., 2003). Notably, the p73 gene is located at 1p36.3 a frequently deleted region in neuroblastomas. This finding suggests that p73 is a promising candidate tumour suppressor gene lost in these tumours (Kaghad et al., 1997). A truncated anti-apoptotic isoform of p73, ΔNp73 lacking the transactivation domain, has been shown to be able to antagonise both p53 and p73. In neuroblastoma, the ΔNp73 isoform has been detected as the predominant form and thus might provide a mechanism of inactivating p53 and p73, particularly because ΔNp73 expression in

neuroblastoma patients is strongly associated with reduced survival (Casciano et al., 2002).

Neuroblastoma cells derive from embryonic tissues that depend on growth factors and it was suggested that IGFs promote proliferation of neuroblastoma cells (El Badry et al., 1991). Analyses of primary neuroblastoma tumour samples showed that 48% expressed IGF-2, but elevated IGF-2 levels did not correlate with prognostic features like disease stage and N-MYC amplification (Sullivan et al., 1995). Despite there is no apparent correlation between prognostic markers and IGF-2 expression, it is noteworthy that neuroblastomas frequently metastasise to tissues that express high levels of IGF-2, such as the bone, liver, skin and lymph nodes. On the other hand, secondary metastases may spread to the lungs and the brain, sites which do not express high levels of IGF-2 (Matthay, 1995). Accordingly, it was suggested that neuroblastoma cells may metastasise to tissues where IGF-2 is delivered in a paracrine fashion and tissues that do not produce IGF-2 are only invaded by aggressive NB cells producing IGF-2 in an autocrine manner (El Badry et al., 1991).

Telomerase is crucial for the maintenance of chromosomal ends and increased telomerase activity is detectable in most cancer cells. The presence of the full-length form of hTERT, a component of telomerase was found to be an independent prognostic marker in neuroblastoma (Krams et al., 2003).

Bcl-2 is highly expressed in a majority of neuroblastoma cell lines as well as primary tumours (Maris and Matthay, 1999). Expression of Bcl-2 in untreated cases of neuroblastoma was associated with unfavorable histology and N-MYC gene amplification (Castle et al., 1993; Oue et al., 1996). Importantly, an inverse correlation between the occurrence of apoptosis and Bcl-2 expression was observed in primary tumours (Oue et al., 1996).

B-MYB is another gene whose expression has been associated with neuroblastoma and as B-MYB is the subject of this thesis the link between B-MYB and neuroblastoma is discussed in more detail in the next section.

1.4 The connection between B-MYB and neuroblastoma

In several cellular systems, B-MYB has been shown to be an important factor that drives cell cycle progression (Sala and Calabretta, 1992; Lin et al., 1994; Raschella et al., 1995; Sala et al., 1996a). Apart from an enhanced proliferation rate, it was demonstrated that B-MYB is able to force certain cell lines to leave quiescence (Sala and Calabretta, 1992; Lin et al., 1994; Sala et al., 1996a; Joaquin et al., 2002; Joaquin and Watson, 2003b) and counteracts signals that induce differentiation of proliferating tissues (Raschella et al., 1995; Bies et al., 1996). In addition, overexpressed B-MYB was associated with a decreased dependence for growth factors (Sala and Calabretta, 1992; Grassilli et al., 1999). Moreover, in certain cell types B-MYB was shown to be required for survival (Santilli et al., 2005) and provides resistance to apoptotic stimuli (Grassilli et al., 1999; Cervellera et al., 2000; Ahlbory et al., 2005; Lang et al., 2005; Santilli et al., 2005).

While the role of B-MYB in cancer has been addressed in several studies, only few reports exist that suggest a connection between B-MYB and neuroblastoma. Raschella and coworkers performed an important study, investigating B-MYB expression in primary neuroblastoma tumour biopsies. From a cohort of 64 neuroblastoma biopsies 33 samples showed B-MYB mRNA expression. While A-MYB and c-MYB mRNA could also be detected in a proportion of neuroblastoma samples, only the expression of B-MYB significantly correlated with increased risk. Interestingly, in samples with N-MYC amplification, expression of B-MYB did not have any further prognostic power, but was significantly associated with lower survival probability in samples with normal N-MYC copy number. This data suggests that B-MYB defines a sub-group of neuroblastoma patients without N-MYC amplification that have a poor survival probability and consequently it was proposed that B-MYB constitutes a prognostic factor that would complement the predictive value of N-MYC (Raschella et al., 1999).
While amplification of the B-MYB locus has been identified in various cancer cell lines and primary tumour biopsies, it has not been reported in neuroblastoma samples as yet (Forozan et al., 2000; Tanner et al., 2000; Zondervan et al., 2000;

Mao et al., 2003). However, gene amplification is not the only mechanism to activate a proto-oncogene and several lines of evidence suggest that aberrant expression of B-MYB could play an important role in the generation or progression of neuroblastoma. Lack of terminal differentiation is one characteristics of cancer and poorly differentiated neuroblastoma tumours are more aggressive. *In vitro* differentiation experiments with neuroblastoma cells revealed that B-MYB mRNA levels successively decrease, but when B-MYB is constitutively over-expressed, neuroblastoma cells fail to differentiate (Raschella et al., 1995). Treatment of neuroblastoma cells with antisense oligonucleotides against B-MYB diminished their growth capacity (Raschella et al., 1995). Whereas ectopic expression of B-MYB enhanced the survival of neuroblastoma cells in response to doxorubicin treatment, inhibition of the B-MYB target gene ApoJ/clusterin renders neuroblastoma cells susceptible to doxorubicin-induced apoptosis (Cervellera et al., 2000). Bcl-2 is another target gene of B-MYB, which is associated with an apoptotic resistant phenotype of cancer cells (Grassilli et al., 1999; Lang et al., 2005). Importantly, expression levels of Bcl-2 are increased in many neuroblastomas (Maris and Matthay, 1999). Moreover, it was proposed that in aggressive neuroblastoma cases, the tumour cells may establish an autocrine IGF production loop (El Badry et al., 1989). B-MYB positively regulates the expression of IGFBP-5, which belongs to a group of proteins that bind insulin-like growth factors (IGFs) with high affinity. Recombinant IGFBP-5 increases the proliferation of neuroblastoma cells in a dose dependent manner and it potentiated the proliferation promoting effect of IGF2 (Tanno et al., 2002).

Treatment failure occurs in all neuroblastoma patient risk-groups, which suggests that the identification and evaluation of additional prognostic markers might further improve the treatment decision. Moreover, while intensified chemotherapy significantly raised the rate and the number of long term neuroblastoma survivors, therapeutic improvements have not substantially ameliorated in the past decade (Westermann and Schwab, 2002). Considering these aspects, I aimed to investigate the role of B-MYB in neuroblastoma.

1.5 Aims of the thesis

With this thesis I aimed to:

- investigate whether targeting B-MYB by RNAi approaches could be of therapeutic value.
- elucidate the mechanism by which B-MYB is stabilised in neuroblastoma cells.
- screen neuroblastoma cell lines and tumour samples for alterations in the B-MYB coding region.
- characterise novel B-MYB variants.
- assess the frequency of the different B-MYB forms in a case-control study.

2 MATERIALS AND METHODS

2.1 Reagents

Chemicals were purchased from VWR (Poole, Dorset, UK), all tissue culture reagents were from Invitrogen (Paisley, UK) and molecular biology enzymes were from Promega (Madison, Wisconsin, USA) unless otherwise stated. Oligonucleotides were purchased from Sigma Genosys (Haverhill, UK). All procedures were carried out at room temperature unless otherwise stated.

2.2 Buffers and solutions

Table 2-1 Buffers and solutions

Buffer or solution	Final concentration of components
Phosphate buffered saline (PBS)	50 mM potassium phosphate, 150 mM NaCl (pH 7.2)
TAE	40 mM Tris-acetate, 1 mM EDTA (pH 8.0)
Hypertonic lysis buffer	1% NP-40, 500 mM NaCl, 20 mM Hepes (pH 7.9), 0.5 mM DTT, 0.2 mM EDTA, 1 x Protease inhibitor cocktail (Roche)
SDS-PAGE sample buffer	45 mM Tris-HCl (pH 6.8), 16% glycerol, 1% SDS, 50 mM DTT, 0.1% bromphenol blue
SDS-PAGE electrophoresis buffer	25 mM Tris-HCl (pH 8.3), 192 mM glycine, 3.5 mM SDS
Western blot transfer buffer	25 mM Tris-HCl (pH 8.3), 19.2 mM glycine, 10% methanol
Ponceau S solution	0.5% ponceau S, 1% glacial acetic acid
Western blot blocking buffer	5% fat free milk powder, 0.1% NP-40 in PBS
Western blot washing buffer	0.1% NP-40 in PBS
Propidium iodide solution	20 μg/ml RNAse, 2 μg/ml propidium iodide (Sigma), 0.1% NP-40 in PBS
Pull down buffer	0.1% NP-40, 40mM NaF, 2mM NaVan, 1 x Protease inhibitor cocktail in PBS
DNA lysis buffer	100 mM Tris-HCl (pH 8.5), 5 mM EDTA, 0.2% SDS, 100 mM NaCl, 0.5 mg/ml proteinase K
Luria-Bertani (LB) broth	1% tryptone, 0.5% yeast extract, 1% NaCl (pH 7.0)
LB-agar	LB broth, 1.5% agar

2.3 Cell biology methods

2.3.1 Cell culture

All cell lines were maintained in Dulbecco's modified Eagle's medium (D-MEM) supplemented with 4 mM L-glutamine and 10% foetal bovine serum (FBS). Non-essential amino acids were added to the medium used with the cell line SK-N-DZ. Cells were incubated at 37°C, 5% CO_2 in a humidified atmosphere. As all cell lines utilised in this study are adherent, cells were washed with PBS and gently trypsinised from the culture vessel prior to use. Cells were pelleted by centrifugation at 300xg at room temperature for 5 mins. This corresponds to 1200 rpm in a Rotina 46R centrifuge (Hettich) and 2400 rpm in an Eppendorf table centrifuge. Cells were counted using a Neubauer counting chamber (Marienfield, Germany).

Table 2-2 Cell lines

Name	Species	Tissue	Disease
SH-SY5Y	human	neural crest	neuroblastoma, from bone marrow
SK-N-AS	human	neural crest	neuroblastoma, from bone marrow
SK-N-DZ	human	neural crest	neuroblastoma, from bone marrow
RH1	human	bone	Ewing sarcoma
RD-ES	human	bone	Ewing sarcoma
WI38	human	lung	normal
HEK-293	human	kidney	transformed with adenovirus
Phoenix A	human	kidney	retrovirus producer cells

2.3.2 Retroviral infection

70-80% confluent Phoenix A cells were transfected with calcium phosphate based methods using the ProFection mammalian transfection system (Promega). In brief, 20 ml medium and 25 μM chloroquine (Sigma) were added to a T75 flask and the

cells were incubated at 37°C. After 30 mins 128 µl CaCl, 20 µg retroviral construct and 1 µg pVSV-vector were mixed in a volume of 1 ml. 1 ml HEPES solution was added to the mix under constant shaking and incubated for 30 mins. 2 ml transfection mixture were added per 20 ml medium and the cells were incubated overnight at 37°C. The following day, 20 ml of fresh medium were added to the cells, which were then incubated for 24 hours at 37°C. The supernatant containing the viral particles was harvested, filtered through a 0.45 µm filter and 4 µg/ml polybrene (Sigma) were added. 2 ml of the viral supernatant were added to a single well of a 6 well plate containing target cells. The plate was spun at 1800 rpm for 45 mins at 32°C and incubated for 4 hours at 37°C. 2 ml fresh viral supernatant were added per well and the plate was spun at 1800 rpm for 45 mins at 32°C. The cells were incubated for 2 hours at 32°C. Then, fresh medium was added to the cells and they were incubated at 37°C overnight. The next day, 2 ml fresh viral supernatant were added per well and the plate was spun at 1800 rpm for 45 mins at 32°C. The cells were incubated for 6h at 32°C.

Table 2-3 Plasmids used for retroviral infections

Name	Insert	Source
Mig- (MSCV-IRES-GFP)	-	(Santilli et al., 2005)
MigDBD	B-MYB DBD	(Santilli et al., 2005)
pSUPER.retro.shB-MYB	shB-MYB	section 2.5.3
pSUPER.retro.shscramble	shscramble	section 2.5.3
pVSV	vesicular stomatitis virus envelope protein	Dr. B. Calabretta

2.3.3 Plasmid transfection

For a 10 cm^2 dish 20 µg plasmid DNA and 20 µl lipofectamine 2000 (Invitrogen) were each diluted in 0.75 ml Optimem. After 5 mins incubation, the diluted DNA was added to the lipofectamine solution and incubated for 20 mins. The DNA-

liposome complexes were mixed with 4.5 ml D-MEM and added to the tissue culture vessel.

For further treatment, cells were replated into 6 well plates at 60% confluence 24 hours after transfection. The cells were incubated for another 24 hours and then subjected to treatments as described in section 2.3.9.

Table 2-4 Plasmids used for transfections

Name	Insert	Tag	Source
pcDNA3.1/His	-	His, Xpress	Invitrogen
pcDNA3.1/His B-MYB	B-MYB	His, Xpress	Dr. A. Sala
pcDNA3.1/His B-MYBG427	B-MYBG427	His, Xpress	section 2.5.4.
pcDNA3.1/His B-MYBM624	B-MYBM624	His, Xpress	section 2.5.4.
CMVFlagB-MYB	B-MYB	Flag	(Johnson et al., 1999)
CMVFlagB-MYB mut15	B-MYB w/o 15 phosphorylation sites	Flag	(Johnson et al., 2002)
CMVGFP-spectrin	GFP-spectrin	-	Clontech
pglMIM-1-luciferase	MIM-1 promoter fused to luciferase	-	(Sala et al., 1999)
CMVrenilla	renilla	-	Clontech

2.3.4 siRNA oligonucleotide transfection

Double stranded siRNA oligonucleotides were purchased from Qiagen. The following sequences were used in RNAi experiments:

siB-MYB: 5'-AAGTCATCGAGCTGGTTAA

Scramble: 5'-CCGAGTATAGAGTACTTAG

Tranfection of RNAi oligonucleotides was essentially carried out as described in section 2.3.3. Normally, RNAi oligonucleotides were applied to cells grown in 12 well plates with 3.5 µl (20 µM) oligonucleotide DNA and 3 µl lipofectamine 2000 in 50 µl Optimem per well. The two solutions were combined and added to 400 µl D-MEM. Cells were harvested between 24 and 72 hours after transfection.

2.3.5 Proliferation assay

After infection and puromycin selection, cells were replated at a density of 5 x 10^3 cells per well in 200 µl D-MEM containing 10% FBS. Cells were counted every other day and the assay was performed in triplicates.

2.3.6 MTS assay

Cells were seeded at a density of 10^3 cells per well of a flat-bottomed 96-well plate and cultured in 100 µl D-MEM containing 10% FBS for 3 days. 20 µl CellTiter 96® Aq_{ueous} One Solution Reagent (Promega) was added to each well according to the manufacturer's instructions. The plates were wrapped in foil and returned to culture at 37°C for 1 hour. After this time, cell viability was measured by reading the absorbance of the triplicates at 490 nm using a 550 Biorad plate-reader.

2.3.7 Quantification of cell death

2.3.7.1 Trypan blue exclusion

The medium supernatant and trypsinised cells from a 12 well plate were combined, pelleted and resuspended in trypan blue (Sigma). The cell suspension was applied into a cell counting chamber and the number of blue cells was visually scored under a microscope.

2.3.7.2 Single cell measurement of genomic DNA fragmentation

Adherent cells were harvested by trypsination and combined with detached cells from the medium supernatant. Cells were pelleted and resuspended in 500 µl PBS. 5 ml precooled 70% ethanol was added drop-wise to the cells while the tube was gently vortexed. Fixed cells were incubated at 4°C for at least 2 hours. Cells were washed once with PBS, resuspended in 500 µl propidium iodide solution and incubated at 37°C for 30 mins. Genomic DNA content was determined with a Beckman Coulter Epics XL flow cytometer (High Wycombe, UK) using an argon laser at 488 nm for excitation. Propidium iodide emission was collected with a filter at 675 nm and GFP emission was measured with a filter at 510 nm.
Typically, 30,000 events were collected and doublet discrimination was used to analyse a single cell population. The analysis was carried out with the Expo32 (Beckman Coulter) software.
Nuclei displaying a hypodiploid, sub-G1 DNA content were identified as apoptotic (Nicoletti et al., 1991). Cell debris, characterised by low forward and side scatter values, was excluded from the analysis.

2.3.8 Immunofluorescence staining

Cells were grown on cover slips, washed with PBS and fixed in 3% paraformaldehyde in PBS at 4°C for 20 mins. Cover slips were washed with PBS and incubated with 0.2% Triton X-100 for 10 mins, rinsed with PBS and incubated with 5% FBS-PBS for 20 mins. Primary antibodies (Table 2-5) were applied in 5% FBS-PBS for 60 mins and then cover slips were washed three times with PBS for 5 mins each before secondary antibodies (Table 2-5) conjugated to a fluorophor were applied in 5% BSA-PBS for 60 mins. Cover slips were washed three times with PBS for 5 mins, then stained with 12.5 µg/ml DAPI (Molecular Probes), mounted onto a microscope slide and sealed. Slides were analysed with an Axioplan 2 microscope (Zeiss) and images were take with SmartCapture VP (Digital Scientific) software.

Table 2-5 Antibodies used for immunofluorescence staining

Antibody	Species	Dilution	Manufacturer
Xpress	mouse	1:500	Invitrogen
Activated caspase-3	rabbit	1:15	BioVision
Anti-mouse IgG FITC	goat	1:200	Sigma
Anti-rabbit IgG TR	goat	1:150	Vector Laboratories

2.3.9 Cell treatments

2.3.9.1 Drug treatments

Cells were treated with 12 μg/ml cycloheximide (Sigma), 5 μM MG-132 (Calbiochem), 20 μM zVAD (MD Biosciences) or 50 μM H_2O_2 (Sigma) for the indicated periods of time. After the addition of the compound, cells were placed into an incubator and cultured at 37°C.

2.3.9.2 Selection

SK-N-AS and SH-SY5Y cells were selected with 5 μg/ml and 2.5 μg/ml puromycin, respectively. The medium containing antibiotics was changed every 3 – 4 days until all mock transfected cells were dead.

2.3.9.3 UV-irradiation

UV-irradiation was performed with direct exposure of the cells to 8 mJ/cm^2 in a UV chamber (BioRad). Following irradiation, cells were returned to a 37°C incubator.

2.4 Protein biology techniques

2.4.1 Immunoblotting

2.4.1.1 Sample preparation

Cultured cells were washed with PBS and disaggregated in hypertonic lysis buffer. Cells were subjected to freeze-thaw cycles and incubated on ice for 20 mins before they were cleared by centrifugation at maximal speed at 4°C. The supernatant was harvested and subjected to protein concentration measurement.

2.4.1.2 Determination of protein concentration

The protein concentration of each sample was measured using the Biorad Protein Assay based on colorimetric properties of Coomassie Brilliant Blue G-250 according to manufacturer's instructions. Absorption was measured using a 550 Biorad plate-reader at 595 nm. A standard curve using known concentrations of BSA was first completed. This allowed calculation of the concentration of total cellular protein in the samples.

2.4.1.3 Western blotting

20-25 μg of protein was diluted to 10 μl with lysis buffer and mixed with 5 μl of 3 x SDS-PAGE sample buffer. The samples were denatured by heating to 100°C for 5 mins, and centrifuged at maximal speed in a microcentrifuge for 3 mins.
For examination of B-MYB, which runs at about 90 kilodaltons, the samples, along with 10 μl of Broad Range Prestained Protein Marker (NEB), were loaded onto a 10% SDS-PAGE mini-gel (Biorad). The proteins were separated in SDS-PAGE electrophoresis buffer by a constant electrical field at 10-20 mA per gel until the bromphenol blue front reached the bottom of the gel. The gel was removed from the glass plates and blotted onto Hybond-C nitrocellulose membrane (Amersham) in a Biorad transfer tank at 90 V for 1 hour using Western blot transfer buffer. For visualising the protein bands, the membrane was stained with Ponceau S solution and destained in H_2O. The membranes were blocked in blocking buffer for 20 mins

and incubated with primary antibodies (Table 2-6) diluted at different concentrations in Western blot blocking buffer. Incubations were for 1 hour at room temperature or overnight at 4°C. Unbound antibodies were removed by three brief washings in Western blot washing buffer, followed by incubation with secondary antibodies (Table 2-6), conjugated to horse-radish-peroxidase (HRP), for 45 mins. The membrane was then washed and the signal was visualised using the ECL Western blotting analysis system (Amersham Biosciences) according to manufacturer's instructions. Autoradiographic films were exposed to the membrane for various periods of time depending on band intensity. Where applicable, bands were quantified using a Biorad Densiometer.

Table 2-6 Antibodies used for Western blotting analysis

Antibody	Species	Dilution	Manufacturer
B-MYB	mouse	1:5	gift of Dr. R. Watson Imperial College London, UK
B-MYB N-19	mouse	1:200	Santa Cruz Biotechnology
PCNA PC10	mouse	1:500	Santa Cruz Biotechnology
Actin I-19	goat	1:500	Santa Cruz Biotechnology
Xpress	mouse	1:5000	Invitrogen
Flag M2 (used w/o detergent)	mouse	1:400	Sigma
Anti-mouse HRP	donkey	1:5000	Amersham Biosciences
Anti-rabbit HRP	donkey	1:5000	Amersham Biosciences
Anti-goat HRP	donkey	1:5000	Santa Cruz Biotechnology

2.4.2 Dual-luciferase reporter assay

For reporter assays, cells were grown and transfected in 12 well plates. Typically, 0.5 μg reporter, which consist of a regulatory sequence fused to the firefly luciferase gene, and 50 ng renilla luciferase expression constructs were transfected

per well with lipofectamine 2000 as described in section 2.3.3. 24 hours after transfection, the growth medium was removed and cells were washed with PBS. Reporter assays were conducted with the dual luciferase kit (Promega) according to manufacturer's instructions. In brief, 100 µl 1 x passive lysis buffer were added and the cells were gently rocked for 15 mins, scraped off the culture vessel and cleared by centrifugation. 50 µl LAR II and 10 µl supernatant were mixed and the firefly luciferase activity was measured with a luminometer. Subsequently, 50 µl Stop & Glo reagent were added and the renilla luciferase activity was measured. Luciferase activity was expressed as the ratio between expression intensities of firefly luciferase and renilla luciferase genes.

2.4.3 Expression and purification of GST-fusion proteins

Bacteria were grown in LB medium at 37°C until OD_{600} was reached. Protein expression was induced with 0.1 mM IPTG for 2 hours at 37 °C. Bacteria were pelleted at 6000xg for 15 mins, resuspended in PBS containing protease inhibitors and sonicated on ice in three intervals for 30 secs at medium power. Cellular debris were pelleted at 12000xg for 10 mins and washed. Glutathion sepharose 4B beads (Pharmacia) were added to the supernatant and incubated for 1h on a wheel. Beads were washed with PBS, containing protease inhibitor cocktail (Roche) and resuspended in PBS also with protease inhibitors.

2.4.4 GST-Pin1 pull down

Cells were lysed in hypertonic lysis buffer and protein concentrations were measured as described in section 2.4.1.2. 200 µg protein lysate were diluted in 1 ml pull down buffer containing GST-fusion proteins and incubated for 1 hour at 4°C on a wheel. Samples were spun at 3000 rpm for 2 mins at 4°C and, after six washes with 1 ml pull down buffer, the supernatant was discarded. The pellet was resuspended in sample buffer, denatured and loaded on a SDS-PAGE mini-gel.

2.5 Molecular biology techniques

Standard protocols, buffers and solutions were from (Sambrook et al., 1989).

2.5.1 Plasmid preparation

A bacterial colony was inoculated in LB broth with appropriate antibiotics overnight at 37°C with vigorous shaking. Small scale plasmid preparation was carried out with a QIAprep Spin Miniprep kit (Qiagen, Crawley, UK) and large scale plasmid preparation was performed using a Plasmid Maxi kit (Qiagen) according to manufacturer's instructions.

2.5.2 Preparation and transformation of chemically competent *Escherichia coli*

For general molecular cloning and plasmid amplification the DH10β strain of *E. coli* (a kind gift from Dr. E. Crescenzi) was used. Chemically competent cells were prepared and transformed according to standard protocols. XL10-Gold bacteria (Stratagene) were used for the generation of pcDNA3.2HisB-MYB variants and transformed according to manufacturer's instructions.

2.5.3 shB-MYB construct cloning

pSUPER.retro.puro (Oligoengine) was cut with BglII and HindIII to linearise the vector. 5'-phosphate groups were removed with calf intestine phosphatase (Roche) and the lineasrised construct was gel purified with the QIAquick Gel extraction kit (Qiagen). The shBMYB and shscramble oligonucleotides were annealed and 5'-phosphate groups were added with the T4 kinase to the oligonucleotides before they were ligated into pSUPER.retro.puro.

shB-MYB: 5'GATCCCCAAGTCATCGAGCTGGTTAATTCAAGAGA
TTAACCAGCTCGATGACTTTTTTTA

shscramble: 5'GATCCCCCCGAGTATAGAGTACTTAGTTCAAGAGA
CTAAGTACTCTATACTCGGTTTTTA

2.5.4 Generation of B-MYB variant constructs

Point mutations were introduced into the pcDNA3.1/His-B-MYB construct with the QuikChange Multi Site Directed Mutagenesis kit (Stratagene). pcDNA3.1/HisB-MYBG427 and pcDNA3.1/HisB-MYBM624 constructs were produced by replacing adenosine at position 1279 with a cytidine in codon 427 (AGC to GGC) and cytidine at position 1872 with a guanosine in codon 624 (ATC to ATG), respectively. Numbering of nucleotides and codons is according to (Nomura et al., 1988).

Primers:
G427: 5'TCCCCTGTCACTGAGAATGGCACCAGTCTGTCCTTCCTG
M624: 5'AGCCTCACCCTGTCAGGTATGAAAGAAGACAACAGCTTG

PCR reaction:
50 ng plasmid DNA were mixed with 100 ng primers, 2.5 µl 10 x QuikChange Multi reaction buffer, 1 µl QuikSolution, 1 µl dNTP mix and 1 µl QuikChange Multi Enzyme blend in a total volume of 25 µl.

Cycling conditions:

1. 95°C for 1 min
2. 95°C for 1 min
3. 55°C for 1 min
4. 65°C for 16 mins
5. 72°C for 10 mins
6. keep at 4°C

Steps 2.-4. were repeated 30 times

Methylase treatment:

Digestion of methylated plasmid DNA was carried out with 1 µl DpnI (10 u/µl) per 25 µl PCR reaction mix at 37°C for 1 hour. XL10-Gold bacteria were directly transformed with Dpn treated plasmid mix according to manufacturer's protocols.

2.5.5 Isolation of genomic DNA

5-10 x 10^6 cells were washed in PBS and lysed overnight in 500 µl DNA lysis buffer containing 0.5 mg/ml proteinase K (Roche) at 55°C. Cell debris was pelleted and supernatant was mixed with 500 µl isopropanol. Genomic DNA was spooled onto a pasteur pipette, washed in 70% ethanol, air dried and resuspended in 100 µl H_2O.

2.5.6 Preparation of RNA and cDNA synthesis

Total RNA was prepared from cell lines for reverse transcription and subsequent PCR based techniques. 3-6 x 10^6 cells were lysed by adding 1 ml of Trizol (Invitrogen) directly to the culture dish. The lysate was mixed with 0.2 ml chloroform by shaking vigorously for 15 secs and then incubated at room temperature for 15 mins. Phases were separated by centrifugation at 12000xg for 15 mins at 4°C. The upper aqueous phase was isolated, mixed with 0.5 ml isopropanol and incubated at room temperature for 10 mins. The RNA was precipitated by centrifugation at 12000xg for 10 mins at 4°C and washed in 70% ethanol. The RNA was then air-dried and re-suspended in 30 µl H_2O.

cDNA was subsequently prepared from 1 μg of total RNA with the SuperScript II RNase H$^-$ Reverse Transcriptase kit (Invitrogen) according to manufacturers instructions using random hexamer primers (Roche).

2.5.7 Quantitative PCR

Q-PCR was carried out using TaqMan™ probe-based chemistry (Applied Biosystems). This principle is based on a fluorogenic oligonucleotide probe consisting of a 5' reporter and a 3' quencher that anneals specifically between the forward and reverse primers.

The PCR reaction was conducted with 1 μl cDNA, 1 μl 20 x primer/probe mix (MYBL2, Hs 00231158_m1 or GAPDH, Hs 99999905_m1 from Applied Biosystems) and 10 μl 2 x TaqMan Universal PCR Master Mix (Applied Biosystems) in a final volume of 20 μl.

The cycling conditions were as follows: an initial incubation at 95°C for 20 secs activated the Amplitaq Gold™ DNA polymerase, followed by 40 cycles composed of a denaturation step at 95°C for 15 secs and an annealing/extension step at 60°C for 1 min. The amplifications were performed using the ABI Prism 7000 Sequence Detection System (Applied Biosystems) and data was analysed using Sequence Detector v 2.2.2 software (Applied Biosystems).

2.5.8 Sequencing of B-MYB

B-MYB was amplified from cDNA as a 1121 base pair 5'- and a 1236 base pair 3'-fragment using either primer pairs NB1 and NB2 or NB3 and NB4 encompassing the entire coding region of B-MYB (illustrated in Figure 3-13A).

Primers:

NB1(F):	5'ATGTCTCGGCGGACGCGCTG
NB2(R):	5'ATCCAGGCGGTACTCGGTCACACT
NB3(F):	5'GAGGCAGCTAACCTCCTCAT
NB4(R):	5'TGACACCCTCAACACCTCAG

PCR reaction:

PCR reactions were performed with the Expand High Fidelity PCR system (Roche) in a final volume of 50 µl with 5 µl 10 x Expand High Fidelity PCR buffer, 1 µl 10 mM dNTPs (Promega), 1 µl NB1 or NB3 and 1 µl NB2 or NB4, 1 µl Expand High Fidelity PCR polymerases and 1 µl DNA.

Cycling conditions:

1. 94°C for 2 mins
2. 94°C for 15 secs
3. 67°C for 30 secs for NB1 and NB2 or
   65°C for 30 secs for NB3 and NB4
4. 72°C for 1 min
5. 72°C for 10 mins
6. keep at 10°C

Steps 2.-4. were repeated 30 - 40 times

The double-stranded PCR product was gel purified (Gel extraction kit, Qiagen) and sent for sequencing at GATC Biotech AG, Konstanz, Germany.
The oligonucleotides employed in the initial PCR reaction were used in combination with the following forward (F) and reverse (R) primers in order to fully and reliably sequence the amplified products (depicted in Figure 3-13A).

Primers:

NB5(F):	5'GGTGGACACAGGAGGCTTCT
NB6(R):	5'CTCAAGAAGCCTCCTGTGTC
NB7(F):	5'GCACCTGGAGGAGGACTTGA
NB8(R):	5'GCAGCACCTCCTTCAAGTCC

2.5.9 Assessment of SNP status in neuroblastoma case-control samples by multiplex capillary heteroduplex analysis (MCHA)

2.5.9.1 Origin of neuroblastoma patient and control samples

DNA samples from neuroblastoma patient biopsies were provided by Dr. Giampaolo Tonini, National Institute for Cancer Research (IST), Italy. DNA control samples were sent by Dr. Licia Iacoviello, Catholic University, Campobasso, Italy and Prof. Bruno Calabretta, University of Modena, Italy. Both patient and contol samples were collected across Italy and are considered to be representative of the Italian population.

2.5.9.2 PCR

Neuroblastoma patient or control sample DNA was amplified and FAM-labelled by PCR using the Expand High Fidelity PCR system as described in section 2.5.8. cDNA or genomic DNA from cell lines with know B-MYB SNP status were prepared and included in the analysis. For cDNA samples, primers were designed for the whole B-MYB coding region. Each primer pair spans a region of around 500 nucleotides and this region overlaps about 50 nucleotides with the adjacent primer pairs (shown in Figure 3-23A). Two primer pairs spanning either exon 8 or exon 13 were used for genomic DNA samples (illustrated in Figure 3-25A).

Primers cDNA:

NB1(F):	5'ATGTCTCGGCGGACGCGCTG
NB1FAM(R):	5'TCCTCCTCGGTCCAGCAAGACTTC
NB13(F):	5'GGACACTGATTGCCAAGCAC
NB13FAM(R):	5'CTGCAAGTTCCTCCTCACTGT
NB25(F):	5'ACAAGGACGGCCTCCAGAGT
NB25FAM(R):	5'GCTGCACTAGGCTGTTGTTGAT
NB27FAM(F):	5'GCTTGGTGTGACCTGAGTAA
NB27(R):	5'CAGCTCCAATGTGTCCTGTT
NB29(F):	5'CCAAGAGCACACCTGTTAAGAC

NB29FAM(R): 5'AGCCAGAGACTTCCGGACTT

NB4FAM(F): 5'ATCATCGAGGACGACATCAG

NB4(R): 5'TGACACCCTCAACACCTCAG

Primers genomic DNA Exon 8:

NB27FAM(F): 5'GCTTGGTGTGACCTGAGTAA

Exon8(R): 5'CCAGCACTGAACACTAGGTGTC

Primers genomic DNA Exon 13:

Exon13FAM(F): 5'CTGAGGCTTGGAAAAGTGTGTATC

Exon13(R): 5'CCTAAGGAAGCAGCATTGAGAC

Cycling conditions:

1. 94°C for 2 mins
2. 94°C for 15 secs
3. 65°C for 30 secs for cDNA primers or
   65°C for 30 secs for Exon 8 primers or
   50°C for 30 secs for Exon 13 primers
4. 72°C for 1 min
5. 72°C for 10 mins
6. keep at 10°C

Steps 2.-4. were repeated 30 - 40 times

2.5.9.3 Heteroduplex formation

PCR products were assessed on an agarose gel. Each sample was transferred with and without reference DNA into a 96 well plate in a final volume of 10 µl.
The samples were denatured at 95°C for 5 mins with 2°C decrements every 2 mins until the base holding temperature of 25°C was reached.

2.5.9.4 Capillary electrophoresis

The MegaBACE 1000 DNA Analyser (Amersham Biosciences) was used for capillary electrophoresis. The capillaries were washed and coated with a 3% linear

polyacrylamide matrix (Amersham Biosciences). Samples were injected into the matrix at a voltage of 3 kV for 30 secs and subsequently electrophoresed at 7 kV for 80 mins.

2.5.9.5 Analysis of heteroduplex data

The Genetic Profiler v.2.2. software (Amersham Biosiences) was used for analysis. Missmatching sense and antisense DNA duplexes (heteroduplexes) migrate slower due to a more open double-stranded configuration under non-denaturing conditions. These heteroduplexes appear as additional peaks compared to the control peak. Samples that generated such double peaks were PCR amplified and sequenced.

2.6 Statistical analysis

Statistical analysis for the comparison of cell death was performed with the paired Student's t-test and QuickCalcs, GraphPad Software was used to analyse the data. The SNP frequency was statistically assessed by the Chi square test performed by Tim Cole, ICH, UCL. A p-value of ≤ 0.05 was considered statistically significant.

3 RESULTS

3.1 Assessing the therapeutic potential of targeting B-MYB by siRNA in neuroblastoma

3.1.1 Background

Solid paediatric tumours are an important cause of childhood death. Among them, neuroblastoma is the most common extracranical malignancy of infancy (Maris and Matthay, 1999). Aggressive chemotherapeutic treatments are applied in advanced stages of neuroblastoma without significantly raising the rate of survival (De Bernardi et al., 2003). This demonstrates the need for new and alternative approaches to treat this disease.

Various cancers, including neuroblastoma, are characterised by augmented expression of certain oncogenic transcription factors that could be used as targets for therapeutic approaches. Aberrant transcription factor activity may lead to abnormal induction of gene pathways causing increased cancer cell survival and/or proliferation. Expression of B-MYB in tumour specimens is associated with poor survival of neuroblastoma patients (Raschella et al., 1999) and exogenous expression of B-MYB can confer drug resistance to neuroblastoma cells *in vitro* (Cervellera et al., 2000). Consistent with this, B-MYB activates genes like Bcl-2 and ApoJ/clusterin that induce an apoptotic-resistant phenotype to cancer cells (Grassilli et al., 1999; Cervellera et al., 2000; Meyer, 2005; Santilli et al., 2005). Suppressing oncogenic transcription factors may render tumourigenic cells susceptible to apoptosis or cause synergistic killing in combination with standard treatments. To abolish B-MYB expression and study the effects of B-MYB downregulation we designed small interfering RNA (siRNA) oligonucleotides targeting different segments of the B-MYB mRNA. We have previously assessed these siRNA oligonucleotides in human fibroblasts and observed increased susceptibility towards apoptosis when B-MYB was downregulated (Santilli et al., 2005).

3.1.2 B-MYB is resistant to siRNA-mediated down-regulation in neuroblastoma cells

To evaluate the effects of B-MYB ablation in paediatric solid tumours, neuroblastoma or Ewing sarcoma cells were cultured for 48 hours with B-MYB or control siRNA oligonucleotides. Ewing sarcoma is another paediatric malignancy that shares several surface markers with neuroblastoma and melanoma tumours and is therefore believed to originate from the neuroectoderm (Lipinski et al., 1987). Cells were harvested and protein lysates were analysed by western blot with a B-MYB antibody. B-MYB protein levels were reduced in RH1 Ewing sarcoma cells in the presence of siRNA directed against B-MYB (Figure 3-1A). However, unlike in Ewing sarcoma cells, B-MYB protein levels were barely changed after treating neuroblastoma cells with siRNAs (Figure 3-1B). Importantly, neuroblastoma SH-SY5Y and Ewing sarcoma RH1 cells express similar levels of B-MYB, which are considerably higher than those observed in normal fibroblasts (Santilli et al., 2005). In addition, other neuroblastoma cell lines such as SK-N-AS or HTLA showed no B-MYB downregulation when cultured with B-MYB siRNA oligonucleotides (data not shown).

Lack of B-MYB degradation in neuroblastoma cells may be due to a defect in the RNA interference-mediated (RNAi) mRNA degradation pathway, whereby, for example, components of the RNA-induced silencing complex (RISC) could not be functional in these cells. Such defects have been observed in *Drosophila* mutants. The *Drosophila* protein Armitage plays a role in RISC assembly and thus in the RNAi process. Armitage mutant cells fail to silence target genes that are endogenously regulated by RNAi (Tomari et al., 2004). To understand whether neuroblastoma cells harbour a defective RNAi pathway, SH-SY5Y cells were transfected with siRNAs and cultured for 48 hours. Total RNA was subsequently prepared and reverse transcribed. B-MYB mRNA expression levels were measured with real time PCR and compared to GAPDH mRNA expression. Despite the lack of B-MYB protein downregulation in neuroblastoma cells, transfection of SH-SY5Y cells with the B-MYB siRNA caused significant reduction of B-MYB mRNA (Figure 3-1C), excluding a general defect in the RNAi-mediated mRNA degradation pathway. Similar to what we observed in fibroblasts (Santilli et al., 2005), B-MYB downregulation in RH1 Ewing sarcoma cells, was accompanied by

increased cell death (Figure 3-1D), thus demonstrating that B-MYB is required for survival of RH1 cells.

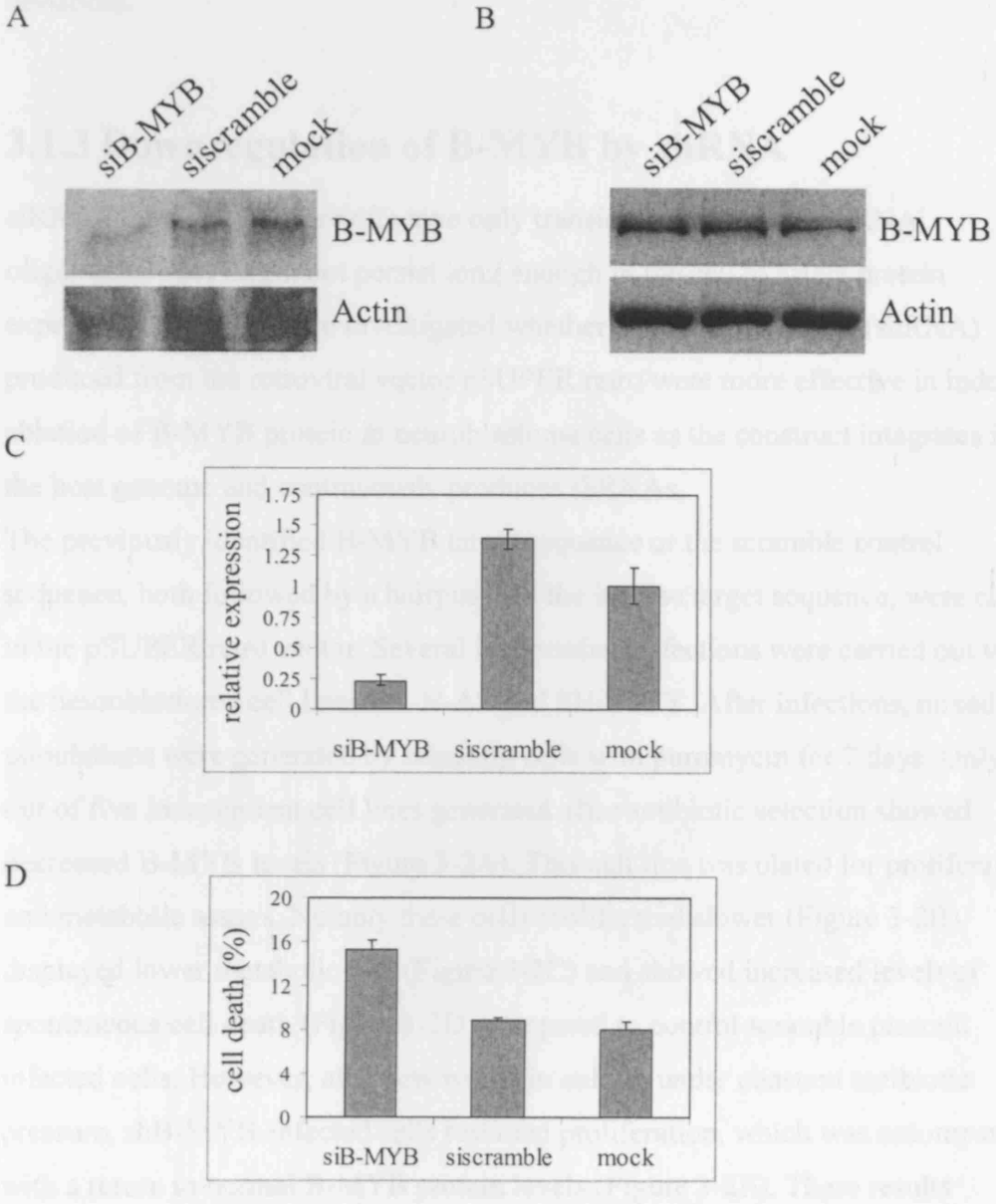


Figure 3-1 Neuroblastoma cells are resistant to siRNA-mediated downregulation of B-MYB

(A) RH1 Ewing sarcoma or (B) SH-SY5Y neuroblastoma cell lines were transfected with scrambled or B-MYB siRNA oligonucleotides. Proteins lysates were quantified, subjected to western blot analysis and B-MYB protein levels were monitored with a B-MYB antibody. Protein loading was verified with an Actin antibody. (C) Neuroblastoma SH-SY5Y cells were treated with siRNA and total RNA was extracted after 48 hours. Levels of

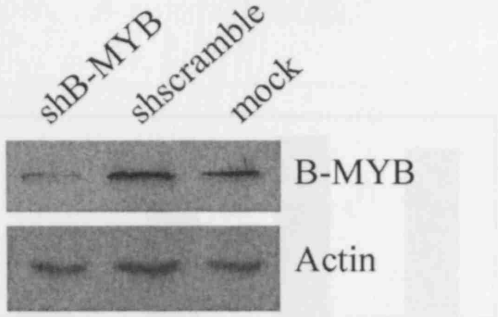
B-MYB mRNA expression relative to GAPDH was assessed by real time PCR. (D) Mock or siRNA transfected RH1 cells were cultured in triplicate wells, harvested and cell death assessed after 48 hours by trypan blue staining and counting. Error bars indicate standard deviations.

3.1.3 Downregulation of B-MYB by shRNA

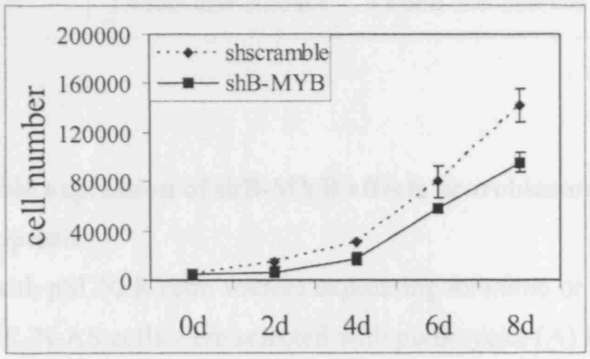
siRNA oligonucleotides are effective only transiently and therefore RNAi oligonucleotides might not persist long enough in the cell to affect protein expression. Therefore, we investigated whether small hairpin RNA (shRNA) produced from the retroviral vector pSUPER.retro were more effective in inducing ablation of B-MYB protein in neuroblastoma cells as the construct integrates into the host genome and continuously produces shRNAs.

The previously identified B-MYB target sequence or the scramble control sequence, both followed by a hairpin- and the inverse target sequence, were cloned in the pSUPER.retro vector. Several independent infections were carried out with the neuroblastoma cell lines SK-N-AS and SH-SY5Y. After infections, mixed populations were generated by selecting cells with puromycin for 7 days. Only one out of five independent cell lines generated after antibiotic selection showed decreased B-MYB levels (Figure 3-2A). This cell line was plated for proliferation and metabolic assays. Notably these cells proliferated slower (Figure 3-2B), displayed lower metabolic rate (Figure 3-2C) and showed increased levels of spontaneous cell death (Figure 3-2D) compared to control scramble plasmid infected cells. However, after few weeks in culture under constant antibiotic pressure, shB-MYB-infected cells resumed proliferation, which was accompanied with a return to normal B-MYB protein levels (Figure 3-2E). These results prompted us to investigate whether B-MYB protein turnover was different in neuroblastoma cells compared to normal or other tumour cell lines, thus potentially providing an explanation for the poor response to RNAi-mediated downregulation.

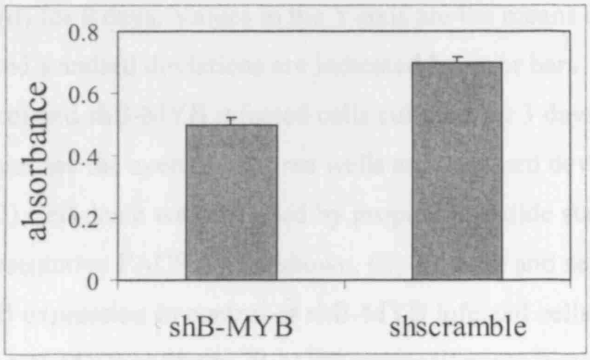
A


shB-MYB
shscramble
mock
B-MYB
Actin

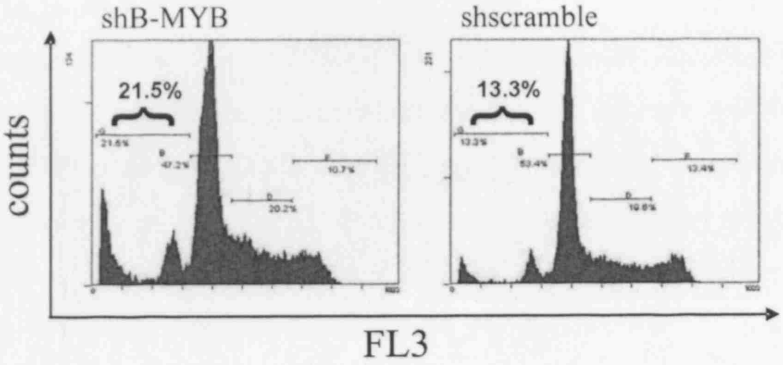
B


200000
160000
120000
80000
40000
0
cell number
shscramble
shB-MYB
0d
2d
4d
6d
8d

C


0.8
0.6
0.4
0.2
0
absorbance
shB-MYB
shscramble

D


shB-MYB
shscramble
21.5%
13.3%
counts
FL3

E

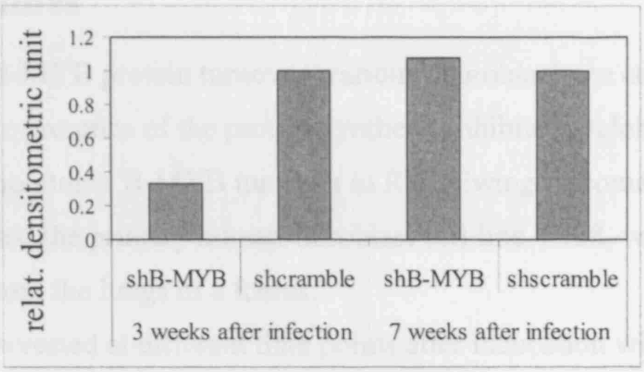


Figure 3-2 Stable expression of shB-MYB affects neuroblastoma cell proliferation and induces apoptosis

After infection with pSUPER.retro vectors expressing scramble or B-MYB shRNAs, neuroblastoma SK-N-AS cells were selected with puromycin. (A) B-MYB protein expression levels were monitored by western blot analysis with a B-MYB antibody. (B) Proliferation of control and shB-MYB cells was monitored by counting the number of cells every other day (d) for 8 days. Values in the Y-axis are the means of the counts from triplicate wells and standard deviations are indicated by error bars. (C) The metabolic turnover of control and shB-MYB infected cells cultured for 3 days was assessed with an MTS assay. Values are the average of three wells and standard deviations are indicated by the error bars. (D) Cell death was analysed by propidium iodide staining and FACS analysis. A representative FACS plot is shown. (E) At three and seven weeks after infection B-MYB expression in control or shB-MYB infected cells was monitored by western blot and a B-MYB antibody. B-MYB expression was quantified by densitometric analysis and normalised to Actin.

3.1.4 Assessing B-MYB protein stability in neuroblastoma cell lines

To measure B-MYB protein turnover, various neuroblastoma cell lines were cultured in the presence of the protein synthesis inhibitor cycloheximide. As a control, we monitored B-MYB turnover in RH1 Ewing sarcoma cells. As a further control we used the primary human fibroblast cell line WI38, which has been established from the lungs of a foetus.

Cells were harvested at different time points after incubation with cycloheximide and protein lysates were analysed by western blotting with a B-MYB antibody (Figure 3-3A). The turnover of B-MYB in 3 different neuroblastoma cell lines was very slow. This was in stark contrast to the short B-MYB protein half life of about 4 hours observed in Ewing sarcoma or fibroblast cells. The B-MYB half life in control cell lines is in good agreement with the B-MYB half life previously observed in 293 HEK cells (Charrasse et al., 2000). This supports the assumption that the slow B-MYB turnover observed in neuroblastoma cells is characteristic of this tumour. Importantly, it should be noted that expression levels of B-MYB in Ewing sarcoma cells are similar or even higher to those observed in neuroblastoma cell lines.

Protein bands in Figure 3-3A were quantified by densitometric analysis. The level of B-MYB expression was normalised against Actin and B-MYB expression at the 0 time point was arbitrarily set to 1 (Figure 3-3B). These graphs show that B-MYB half life in the neuroblastoma cell lines SH-SY5Y, SK-N-AS and SK-N-DZ is between 24-48 hours, compared to the half life of about 4 hours detected in normal fibroblasts or Ewing sarcoma cells.

To assess specificity, we monitored the turnover of PCNA, a nuclear protein ubiquitously expressed by proliferating mammalian cells. The blots were reprobed with a PCNA antibody and protein band intensities were measured by densitometric analysis. Calculations were performed as described above. The turnover of PCNA in neuroblastoma and the Ewing sarcoma cell lines was similar (Figure 3-3A, C). This shows that the observed differences in B-MYB degradation kinetics are not due to a non-specific defect of the protein turnover machinery in neuroblastoma cells.

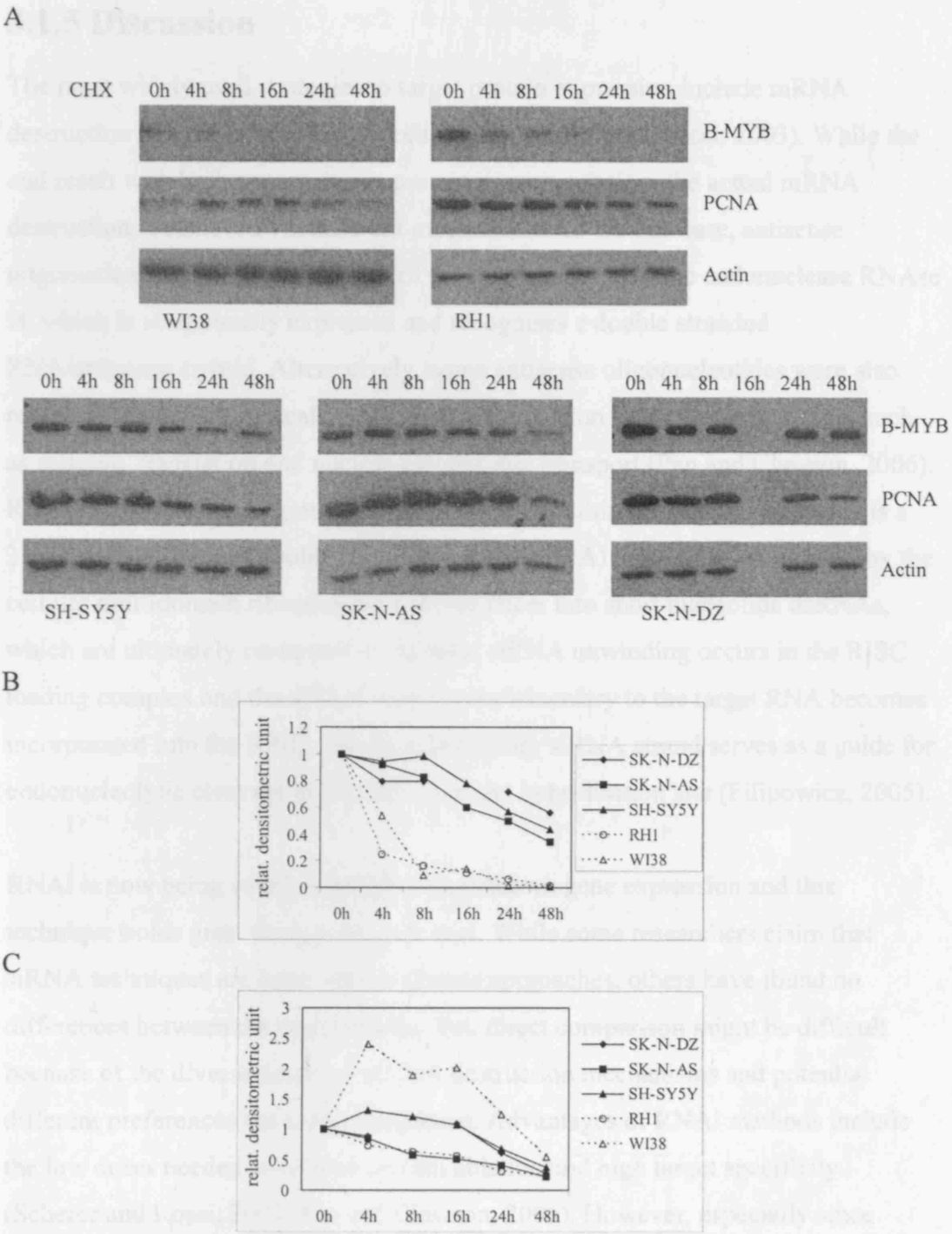


Figure 3-3 B-MYB protein displays an increased half life in neuroblastoma cell lines

(A) Several cell lines were cultured in the presence of cycloheximide (CHX) for the indicated times. Equal amounts of proteins lysates were subjected to western blot analysis with the indicated antibodies. (B) B-MYB or (C) PCNA expression levels were quantified by densitometric analysis. Relative B-MYB or PCNA levels were normalised with the values of the corresponding Actin bands.

3.1.5 Discussion

The most widely used strategies to target protein expression include mRNA destruction by antisense or RNAi techniques (Scherer and Rossi, 2003). While the end result with both approaches is protein downregulation, the actual mRNA destruction is achieved via different mechanisms. In the one case, antisense oligonucleotides provoke cleavage of the target mRNA by the endonuclease RNAse H, which is ubiquitously expressed and recognises a double stranded RNA/antisense hybrid. Alternatively, some antisense oligonucleotides were also reported to interfere sterically with protein expression by blocking processes such as splicing, translation and nuclear-cytoplasmic transport (Pan and Clawson, 2006). RNAi is a gene-silencing mechanism in which the ultimate effector molecule is a 21-23 siRNA. Longer double stranded RNA (dsRNA) molecules are cleaved by the cellular multidomain ribonuclease enzyme Dicer into short nucleotide dsRNAs, which are ultimately processed to siRNAs. siRNA unwinding occurs in the RISC loading complex and the siRNA strand complementary to the target RNA becomes incorporated into the RISC. The complementary siRNA strand serves as a guide for endonucleolytic cleavage of the mRNA at the hybridisation site (Filipowicz, 2005).

RNAi is now being widely applied to knockdown gene expression and this technique holds great therapeutic potential. While some researchers claim that siRNA techniques are better than antisense approaches, others have found no differences between the two methods. Yet, direct comparison might be difficult because of the diverse nature of mRNA destruction mechanisms and potential different preferences for a target sequence. Advantages of RNAi methods include the low doses needed to achieve protein ablation and high target specificity (Scherer and Rossi, 2003; Pan and Clawson, 2006). However, especially since RNAi techniques are still fairly novel, more work is needed to elucidate whether employing one method over the other could be advantageous.

In the past, B-MYB ablation has been mainly achieved by antisense techniques, which pointed to an involvement of B-MYB function in cell proliferation. Arsura and colleagues were the first to apply antisense oligonuclotides against B-MYB, which consequently led to inhibited proliferation of lymphoid and myeloid cell

lines (Arsura et al., 1992). Likewise, the clonogenic growth capacity was significantly lower in antisense transfected fibroblast and neuroblastoma cells (Sala and Calabretta, 1992; Raschella et al., 1995) and microinjection of B-MYB antisense oligonucleotides resulted in a decreased number of glioblastoma cells entering S-phase (Lin et al., 1994). Overall, these experiments indicate that reduction of B-MYB expression impairs normal cell proliferation in a variety of different cell types, but they do not specify whether this effect is due to cell cycle progression defects or impaired survival.

The mechanism leading to decreased proliferation in the absence of B-MYB was investigated in more detail with both RNAi and antisense techniques. Cell cycle profiles of fibroblasts transfected with siRNAs against B-MYB reveal a partial cell cycle block at the G2/M check point as well as increased cell death (Santilli et al., 2005). An elevated apoptotic response was also observed in leukaemia cells with antisense approaches, whereby apoptosis was presumably induced via direct transcriptional regulation of Bcl-2 by B-MYB (Lang et al., 2005). Neither cell cycle arrest nor apoptosis were detected in T98G ganglioblastoma cells when B-MYB expression was abolished by RNAi. This is particularly surprising, as RNAi treatment against B-MYB inhibited expression of the cell cycle-associated proteins cyclin B1 and cdc2 (Zhu et al., 2004).

These findings were opposed by observations with cultured neuronal PC12 cells, sympathetic neurons, and cortical neurons, whereby downregulation of B-MYB provided substantial protection against death elicited by either NGF withdrawal or camptothecin exposure (Liu et al., 2004). Such contrasting observations could reflect distinct requirements for B-MYB in different cell systems, particularly when comparing primary versus transformed cells. Nevertheless, application of B-MYB antisense and siRNA constructs at higher concentrations induced death in untransformed neuronal cells (Liu et al., 2004). It thus appears that particularly in primary neuronal cells, B-MYB levels are carefully balanced and even slight deviations from the normal degree of B-MYB expression have severe consequences for cell survival.

At least in certain circumstances, B-MYB downregulation has been proven to affect gene expression as well as proliferation by inducing growth arrest and/or cell death. These findings suggest that interfering with B-MYB expression might keep

uncontrolled proliferation of transformed cells in check and thus provides an attractive therapeutic tool to target transformed cells.

In the light of these findings, we sought to determine whether targeting B-MYB by RNAi approaches in neuroblastoma cells as well as other cancer cell lines derived from the neuroectoderm would also impair proliferation and elucidate by what mechanism this is achieved. We have previously tested different target sequences for RNAi-mediated downregulation of B-MYB and successfully silenced gene expression in mortal, human fibroblasts (Santilli et al., 2005). Surprisingly, downregulation of the B-MYB protein could not be accomplished in a number of neuroblastoma cell lines with siRNA oligonucleotides.

We reasoned that the relatively high expression of B-MYB in neuroblastoma cells might hinder effective downregulation, as B-MYB expression in the fibroblast cell line previously used for siRNA experiments is low. Thus we tested the downregulation capacity of the B-MYB siRNAs in Ewing sarcoma cells. Ewing cells typically harbour a t(11;22)(q23-24;q12) translocation and expression of specific surface antigens suggests that they are related to the neuroectodermal lineage. Among these surface markers are the ganglioside GD2, the neural cell adhesion molecule N-CAM and the nerve growth factor (NGF) receptor (Lipinski et al., 1987). Although the Ewing sarcoma cell line RH1 exhibits high B-MYB expression levels and similar to the ones in neuroblastoma cells, silencing of B-MYB could be reached efficiently.
A key finding was that even though diminished B-MYB protein expression could not be detected in neuroblastoma cells, treating them with siRNA oligonucleotides readily reduced the B-MYB transcript. This observation confirms that the RNAi pathway is functional in neuroblastoma cells and it raises the question why B-MYB protein ablation could not be achieved in neuroblastoma cells.

Despite B-MYB downregualtion with constitutive RNAi techniques proved difficult, we managed to obtain one neuroblastoma cell line with reduced B-MYB protein expression levels. This cell line proliferated slower compared to the control cells, which was reflected in lower metabolic turnover. These observations confirm previous results by Raschella and coworkers where antisense-mediated B-MYB

ablation hampered proliferation of the neuroblastoma cell line Lan-5 (Raschella et al., 1995). This is particularly important as Burgess et al. have observed that antisense molecules with four contiguous guanosines can interfere with cell proliferation in an unspecific manner (Burgess et al., 1995).

Although, shRNAs delivered by retroviral vectors are meant to stably integrate into the genome, downregulation of B-MYB was lost after further culturing the cells for several weeks. Presumably, the return to normal B-MYB expression levels in shB-MYB infected SK-N-AS cells was accompanied by genomic rearrangements, similar to that observed when Lan-5 clones were analysed after treatment with antisense constructs and subsequent antibiotic selection. In this study, rearrangements in the antisense B-MYB insert were detected, which most probably prevented its expression and consequential protein ablation, thus enabling the cells to grow (Raschella et al., 1995).

An important finding was that neuroblastoma and Ewing sarcoma cells with decreased B-MYB levels displayed augmented spontaneous apoptosis, confirming similar findings in human fibroblasts and transformed haematopoietic cells (Lang et al., 2005; Santilli et al., 2005). Accordingly, our results support the notion that reduced proliferation observed in different cell systems after B-MYB silencing is due to increased apoptosis and particularly that this is the case for transformed cells derived from the neuroectoderm. Therefore, this suggests that B-MYB is an important regulator of cell survival in addition to its already widely acknowledged properties to regulate cell cycle progression. Unlike in human fibroblasts, neuroblastoma cells did not arrest at the G2/M cell cycle check point.

Since we have clearly accomplished B-MYB mRNA destruction without any consequences for B-MYB protein expression, we considered that B-MYB might be aberrantly stabilised in neuroblastoma cells. Indeed, several mechanisms have been described that result in increased protein stabilisation of other transcription factors independently of transcriptional effects (Bies and Wolff, 1997; Gavine et al., 1999). We found that the B-MYB half life was greatly increased in neuroblastoma cell lines, which were refractory to B-MYB ablation by RNAi.

Oncogenic transformation was associated with deregulated proteolysis in various Burkitt's lymphoma B-cells, whereby the proteasome machinery was defective due to upregulation of deubiquitinating enzymes (Gavioli et al., 2001). However, general impairment of proteolytic pathways is an unlikely scenario in our study, because degradation of PCNA, another proliferation-associated protein was not delayed in neuroblastoma cells in comparison to the control cell lines.

One of the rationales behind assessing the downregulation capacity of B-MYB in neuroblastoma cells was to be able to translate our findings into a potential therapeutic application. One of the major obstacles with antisense- and RNAi-based methods to date is efficient delivery to particular organs or tissues *in vivo*. Specific delivery of siRNAs to particular tissues is thought to raise therapeutic efficacy, reduce siRNA concentration and minimise off-target effects.
Some tissues of the body are more readily accessible than others and may thus be easier to target. The respiratory tract for example is easily accessible and intranasal application of siRNAs against subunits of the viral RNA-dependent RNA polymerase has prevented mice from infection with parainfluenza virus. Strikingly, cells of the murine respiratory system have integrated siRNAs with or without transfection reagents (Bitko et al., 2005).
However, cells of most other tissues do not take up siRNAs in the absence of transfection reagents and particularly for tissues deep within the body, different delivery methods are being evaluated. Such an alternative approach was performed with adenovirus encoding RNAi against hypoxia-inducible factor-1α. Viral particles were directly injected into murine tumour masses and this treatment sensitised tumours to ionizing irradiation, consequently reducing tumour growth when RNAi-mediated downregulation was combined with irradiation (Zhang et al., 2004).
When no direct injection is practicable, other efforts have been undertaken to deliver RNAi in a tissue-specific manner. Such an alternative strategy includes liposomal encapsulation and this is believed to increase stability in blood as well as cellular incorporation. Such an approach has been used to deliver c-MYB antisense oligonucleotides to mice engrafted with human neuroblastoma cells. Moreover, directing these liposomes to cells of neuroectodermal origin by incorporating

antibodies against the GD2 disganglioside greatly increased survival of mice engrafted with human neuroblastoma cells (Brignole et al., 2004).
Another attempt would be to couple siRNAs to a fusion protein involving a targeting antibody fragment linked to protamine. siRNA oligonucleotides could be readily anchored to the fusion protein due to the nucleic acid-binding properties of protamine and specifically target tissues that harbour surface markers recognised by the antibody fragment (Song et al., 2005).
The latter two approaches seem particularly promising to target oncogenic transcription factors in neuroblastoma. However, overall our results revealed that B-MYB may not be a good target for therapeutic downregulation in neuroblastoma tumours due to the very long half life of B-MYB in neuroblastoma cells. However, we have gathered *in vitro* evidence suggesting that this strategy could be successfully applied to specifically kill Ewing sarcoma tumours. To target Ewing sarcoma tumours, antibodies against the surface antigen MIC2 (CD99) could be linked to the liposome as MIC2 is highly expressed by Ewing sarcoma and other primitive neuroendocrine tumours (Sandberg and Bridge, 2000).

Generally, our results confirm previous findings in neuroblastoma cells with antisense techniques. This internally validates the two methods to downregulate B-MYB and suggests that decreased proliferation of neuroblastoma cells is indeed due to B-MYB downregulation. Our findings also imply that lack of B-MYB expression in neuroblastoma cells elicits an apoptotic response.

3.2 Evaluating the biological consequences of increased B-MYB protein stability

3.2.1 Background

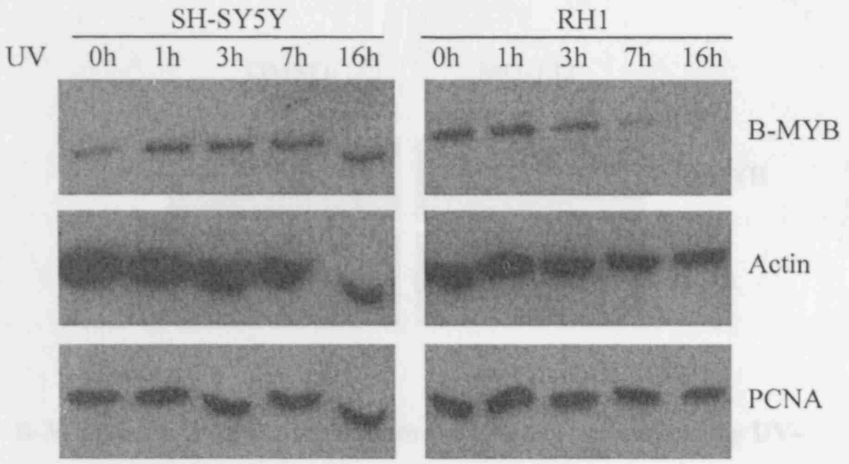
v-MYB is the only MYB family member directly implicated in tumourigenesis. As a component of oncogenic retroviruses, v-MYB is able to transform avian haematopoietic cells (Oh and Reddy, 1999). While c-MYB is commonly truncated through retroviral insertion resulting in avian T and B cell lymphomas as well as murine myeloid leukaemia (Weston, 1999), A-MYB transgenic mice display hyperplasic B-cell expansion in the spleen and lymph nodes (DeRocco et al., 1997). Similarly, a potential direct role of B-MYB in cancer still remains to be established. However, B-MYB was found to be aberrantly expressed or amplified in different human tumours (Tanner et al., 2000; Forozan et al., 2000; Skotheim et al., 2002; Bar-Shira et al., 2002) and its expression has been linked to advanced stages of neuroblastoma (Raschella et al., 1999).

Abnormal gene expression may result in deregulated cellular control ultimately facilitating malignant transformation. Typically, increased protein levels result from gene amplification or deregulated transcription. Aberrant protein stabilisation could have the same effect as the protein might accumulate and therefore occur at abnormal expression levels. In fact, previous studies have suggested that protein stabilisation could contribute to tumourigenesis (Treier et al., 1994; Tsurumi et al., 1995; Gavine et al., 1999). Therefore, we hypothesise that abnormal B-MYB stabilisation could confer neuroblastoma cells with a growth advantage that might contribute to aggressive behaviour. This theory is supported by the fact that B-MYB overexpression in LAN-5 neuroblastoma cells significantly increased resistance to cell death induced by doxorubicin (Cervellera et al., 2000) and rendered the cells refractory to differentiation (Raschella et al., 1995).

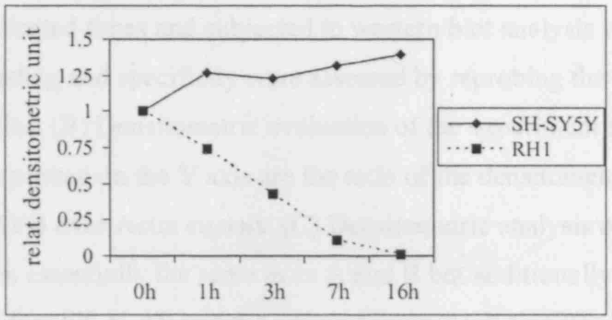
3.2.2 Increased B-MYB stability in UV-irradiated neuroblastoma cells

To assess whether increased B-MYB stability in neuroblastoma cell lines could be of physiological importance we tested different stimuli in neuroblastoma as well as Ewing sarcoma cell lines and assessed B-MYB degradation by western blot.
We measured B-MYB expression levels and cell death in response to UV-irradiation. UV-irradiation causes DNA damage, apoptosis and protein destabilisation and it has previously been reported that disruption of the B-MYB gene sensitises cells to UV-induced apoptosis (Ahlbory et al., 2005). We thought it would be interesting to assess whether B-MYB stability was changed in cells exposed to UV-irradiation. To this end, we exposed Ewing sarcoma or neuroblastoma cell lines to 8 mJ/cm^2 UV-irradiation and harvested the cells for protein analysis at different time points after the treatment. While B-MYB protein levels sharply declined following UV-irradiation in RH1 cells, they were unchanged in SH-SY5Y cells (Figure 3-4A, B). As a control, we monitored expression levels of PCNA by reprobing the blot with a PCNA specific antibody. PCNA turnover was similar in irradiated RH1 and SH-SY5Y cells (Figure 3-4A). In further experiments, a stable B-MYB protein was also observed with the neuroblastoma cell line SK-N-AS. Interestingly, B-MYB was rapidly destroyed after UV-treatment of the Ewing sarcoma cell line RD-ES (Figure 3-4C).
To understand whether rapid down-modulation of B-MYB in response to UV-irradiation in Ewing sarcoma cells occurs at the mRNA or protein level, total RNA was extracted from RH1 cells at different time points after UV-irradiation. Real time PCR confirmed that downregulation of B-MYB occurs at the protein level, since B-MYB mRNA expression is constant in RH1 cells exposed to UV (Figure 3-4D). Next, RH1 cells were grown in the presence of the proteasome inhibitor MG-132 or the solvent DMSO prior to UV-irradiation. Inhibition of the proteasome prevented B-MYB depletion in RH1 cells (Figure 3-4E), suggesting that the proteasome machinery is required for regulating B-MYB levels in response to UV-stress.

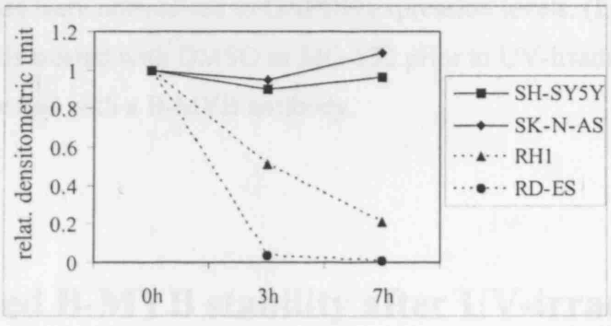
A


SH-SY5Y
RH1
UV
0h 1h 3h 7h 16h
0h 1h 3h 7h 16h
B-MYB
Actin
PCNA

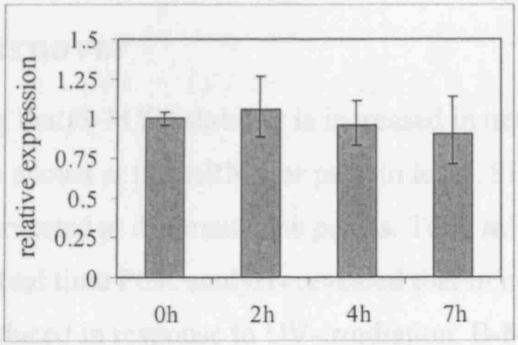
B


relat. densitometric unit
1.5
1.25
1
0.75
0.5
0.25
0
0h 1h 3h 7h 16h
SH-SY5Y
RH1

C


relat. densitometric unit
1.2
1
0.8
0.6
0.4
0.2
0
0h 3h 7h
SH-SY5Y
SK-N-AS
RH1
RD-ES

D


relative expression
1.5
1.25
1
0.75
0.5
0.25
0
0h 2h 4h 7h

E

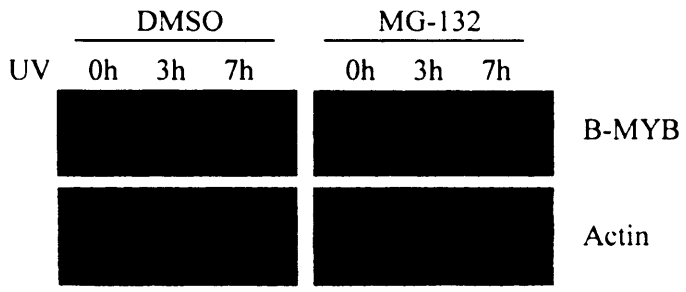


Figure 3-4 B-MYB is resistant to proteasomal degradation induced by UV-irradiation in neuroblastoma cells

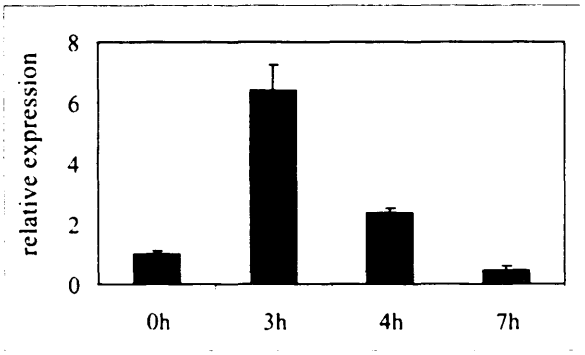
(A) Protein lysates from UV-treated neuroblastoma and Ewing sarcoma cells were harvested at the indicated times and subjected to western blot analysis with a B-MYB antibody. Equal loading and specificity were assessed by reprobing the blots with Actin and PCNA antibodies. (B) Densitometric evaluation of the experiment shown in panel A. Relative values are plotted on the Y axis are the ratio of the densitometric readings obtained from B-MYB over Actin signals. (C) Densitometric analysis of a western blot. The experiment was essentially the same as in A and B but additionally included the neuroblastoma cell line SK-N-AS and the Ewing sarcoma cell line RD-ES. (D) RH1 cells were UV-irradiated and RNA was extracted at the indicated time points. cDNA was prepared and B-MYB mRNA expression was measured by real time PCR. B-MYB expression intensities were normalised to GAPDH expression levels. (E) Western blot analysis of RH1 cells treated with DMSO or MG-132 prior to UV-irradiation. Expression of B-MYB was detected with a B-MYB antibody.

3.2.3 Increased B-MYB stability after UV-irradiation is a combined effect of transcription induction and protein turnover

Following the finding that B-MYB stability is increased in neuroblastoma cells, we assessed whether this occurs at the mRNA or protein level. SH-SY5Y cells were UV-irradiated and harvested at different time points. Total mRNA was isolated and reverse transcribed. Real time PCR analysis revealed that in neuroblastoma cells the B-MYB mRNA is induced in response to UV-irradiation. B-MYB mRNA levels,

normalised to GAPDH, are displayed in Figure 3-5A. When cells were pretreated with cycloheximide 1 hour before UV-irradiation, B-MYB protein levels declined marginally in neuroblastoma cells, whereas again declined rapidly in RH1 cells after UV-irradiation (Figure 3-5B). This suggests that prolonged B-MYB protein levels after UV-treatment in neuroblastoma cells were not only due to *de novo* translation of B-MYB as cycloheximide blocks protein synthesis.
Overall, these results show that the B-MYB mRNA is induced and that the protein is stabilised in neuroblastoma cells following UV-irradiation.

A



B

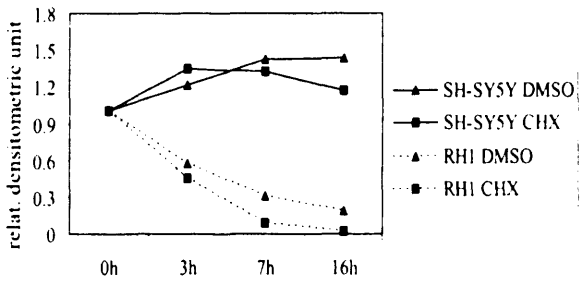


Figure 3-5 B-MYB mRNA is induced after genotoxic damage
(A) SH-SY5Y cells were UV-irradiated and cells were collected for RNA extraction. Total cell RNA was reverse transcribed and subjected to real time PCR analysis. B-MYB transcript levels were normalised to GAPDH expression. (B) Cells were cultured in the presence of cycloheximide (CHX) or DMSO and subjected to UV-exposure. Western blot analysis with an antibody directed against B-MYB was performed. Protein bands were measured by densitometric analysis and B-MYB expression levels are displayed normalised to the Actin signal.

3.2.4 Neuroblastoma cells are resistant to UV-induced apoptosis

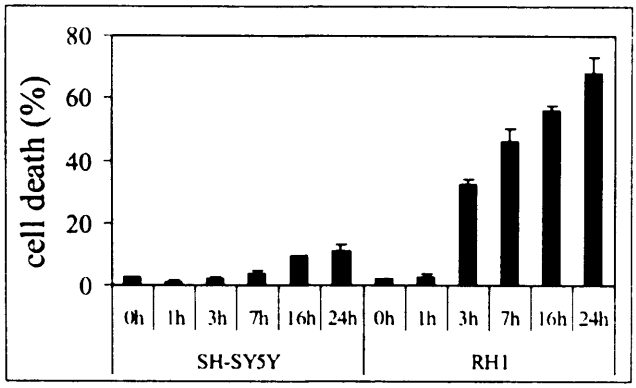
DNA damage induced by UV-irradiation activates tumour suppressors such as p53, which allow the cell to repair DNA damage or ultimately induce cell death (Latonen and Laiho, 2005). To assess the effect of UV-irradiation, cell cycle profiles of neuroblastoma and Ewing sarcoma cells were compared. Propidium iodide staining and FACS analysis demonstrated that the Ewing sarcoma cells RH1 and RD-ES, were highly sensitive to UV-irradiation, showing a much greater degree of apoptosis compared to neuroblastoma cells (Figure 3-6A, B).

While the subG1 peak increased over time in UV-irradiated RH1 cells, cell cycle profiles disclosed that the number of cells in G0/1, S and G2/M phase of the cell cycle declined (Figure 3-6C). The percentage of cells in S or G2/M phase declined more rapidly than the number of cells in G0/1 phase. This suggests that a proportion of UV-irradiated RH1 cells exit the cell cycle and arrest in G0.

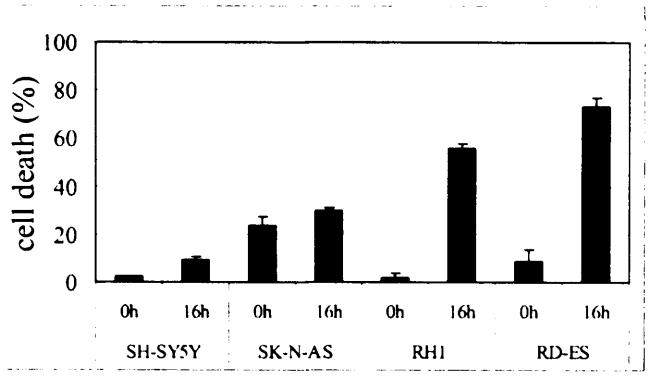
To test whether reduced B-MYB levels in RH1 cells in response to UV-irradiation were due to increased apoptosis, cells were cultured with the pan-caspase inhibitor z-VAD before irradiation. Subsequently, cells were lysed at the indicated times and subjected to western blot analysis with a B-MYB antibody. B-MYB levels declined quickly even in the presence of z-VAD (Figure 3-7A). Loading was assessed by reprobing the blot with an antibody specifically recognising Actin. Inhibition of apoptosis by z-VAD was confirmed by measuring subG1 cells by FACS analysis (Figure 3-7B). This experiment implies that B-MYB degradation is not a mere consequence of apoptosis.

Increased B-MYB stability correlates with the observation that neuroblastoma cells tolerate UV-induced genotoxic damage much better than Ewing sarcoma cell lines analysed in this study. This prompted us to ask the question whether B-MYB stabilisation or overexpression could confer protection from UV-induced apoptosis.

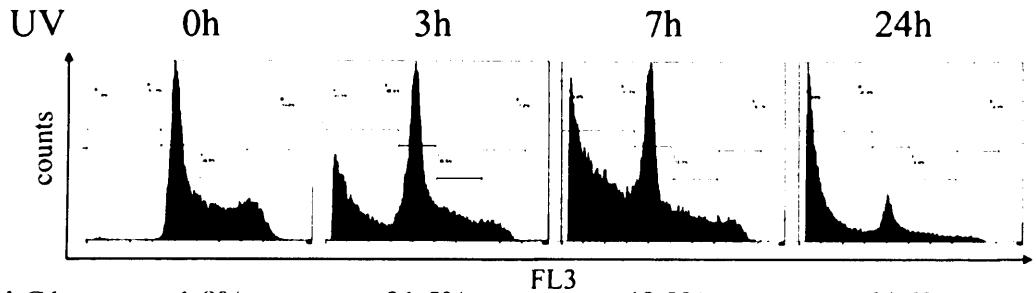
A



B



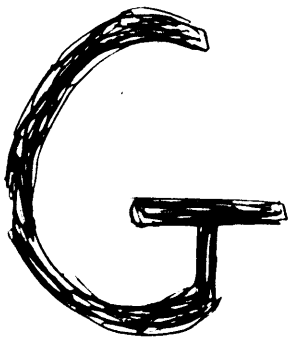
C



subG1	1.8%	31.5%	48.9%	64.6%
G0/1	51.4%	42.9%	31.3%	20.0%
S	28.0%	16.5%	12.1%	9.4%
G2/M	14.6%	7.2%	6.0%	4.1%

Figure 3-6 Neuroblastoma cells are resistant to UV-induced apoptosis

(A) Neuroblastoma SH-SY5Y and Ewing sarcoma RH1 cell lines were exposed to UV-irradiation and cell death was scored by counting cells showing hypodiploid DNA content by FACS analysis. (B) The same procedure as in A but additionally including the neuroblastoma cell line SK-N-AS and RD-ES Ewing cells. Cell death was scored 16 hours after UV-irradiation. (C) Representative cell cycle profiles of UV-irradiated RH1 cells. The numbers indicate the percentage of gated cells in the respective cell cycle phases.



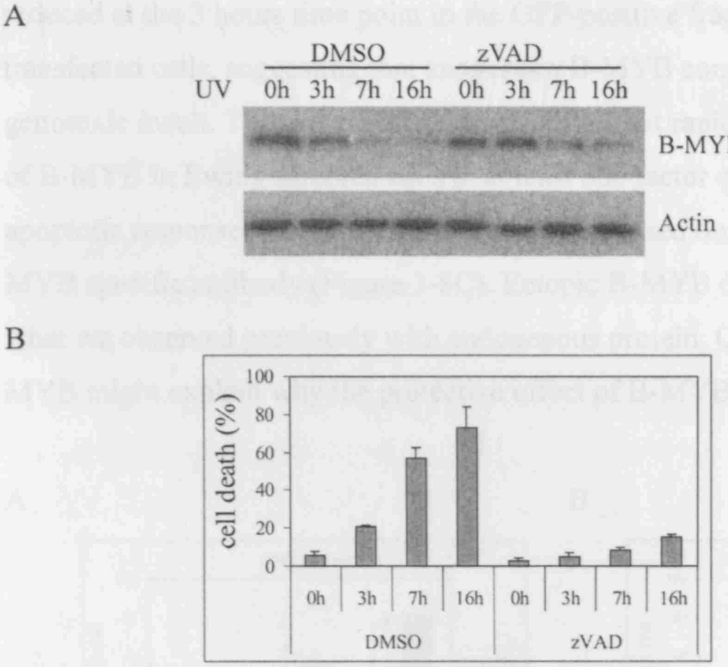


Figure 3-7 UV-irradiation induces B-MYB degradation in the absence of apoptosis
(A) RH1 cells were UV-irradiated in the presence or absence of the pan-caspase inhibitor zVAD and degradation of B-MYB was monitored by western blotting with a B-MYB antibody. (B) Assessment of apoptosis in cells cultured with or without zVAD. Cell death was scored by propidium iodide staining and FACS analysis.

3.2.5 Effect of B-MYB overexpression in UV-sensitive Ewing sarcoma cells

The rapid induction of apoptosis in UV-treated Ewing sarcoma cells is associated with B-MYB protein degradation. To investigate whether apoptosis could be caused by B-MYB depletion, we transiently overexpressed B-MYB, or an empty control plasmid, in RH1 cells along with a GFP expression vector at a ratio of 5:1. Overexpression of B-MYB has been shown to have a protective effect in other cell systems against apoptosis evoked by DNA damaging agents (Grassilli et al., 1999). Cells were subjected to UV-irradiation and apoptosis was monitored by propidium iodide staining and FACS analysis of the whole cell population (Figure 3-8A) as well as of GFP-positive cells (Figure 3-8B). As expected, apoptosis was rapidly induced in control vector transfected cells. Notably, the rate of cell death was

reduced at the 3 hours time point in the GFP-positive fraction of B-MYB transfected cells, suggesting that exogenous B-MYB conferred protection to genotoxic insult. This experiment demonstrates that rapid proteasomal degradation of B-MYB in Ewing sarcoma cells is at least one factor contributing to the apoptotic response. Exogenous B-MYB was analysed on a western blot with a B-MYB specific antibody (Figure 3-8C). Ectopic B-MYB declines rapidly, similar to what we observed previously with endogenous protein. Quick destruction of B-MYB might explain why the protective effect of B-MYB was only transient.

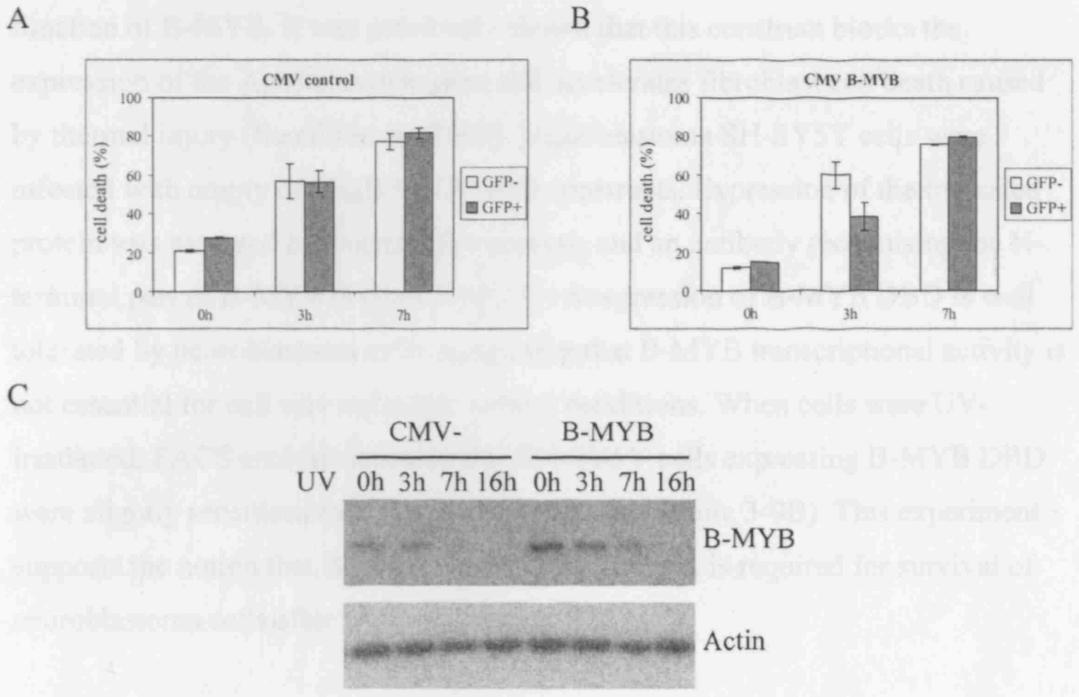


Figure 3-8 Overexpression of B-MYB transiently protects Ewing sarcoma cells from UV-induced cell death

RH1 cells were transfected with empty or B-MYB CMV vectors along with a GFP plasmid and exposed to UV-irradiation. For each time point cell death was monitored by propidium iodide staining and FACS analysis. (A) Percentages of cell death of the whole cell population. (B) GFP-positive cells were gated and percentages of apoptosis were displayed on the y-axis. Error bars show the standard deviations. Student's t-Test analysis shows that the protective effect of CMV-B-MYB at the 3h time point is significant ($p=0.017$; $n=4$). (C) Western blot analysis of B-MYB expression at the indicated time points following UV-irradiation. Note that exogenous, like endogenous, B-MYB is quickly destroyed following UV-irradiation.

3.2.6 Use of a dominant-negative B-MYB construct to suppress B-MYB target gene expression in neuroblastoma cells

Having established the protective role of B-MYB in genotoxic stress, we wanted to assess whether persistent B-MYB levels in UV-treated neuroblastoma cells is involved in their apoptotic-resistant phenotype. Since downregulating B-MYB by RNAi approaches is difficult in neuroblastoma cells (see chapter 3.1), we used a transcriptional dominant-negative B-MYB (B-MYB DBD) construct to assess the function of B-MYB. It was previously shown that this construct blocks the expression of the ApoJ/clusterin gene and accelerates fibroblast cell death caused by thermal injury (Santilli et al., 2005). Neuroblastoma SH-SY5Y cells were infected with empty or MigB-MYB DBD constructs. Expression of the truncated protein was assessed by western blot analysis and an antibody recognising the N-terminal part of B-MYB (Figure 3-9A). Overexpression of B-MYB DBD is well tolerated by neuroblastoma cells, suggesting that B-MYB transcriptional activity is not essential for cell survival under normal conditions. When cells were UV-irradiated, FACS analysis revealed that SH-SY5Y cells expressing B-MYB DBD were slightly sensitised to UV-induced apoptosis (Figure 3-9B). This experiment supports the notion that B-MYB, among other factors, is required for survival of neuroblastoma cells after DNA damage.

A B

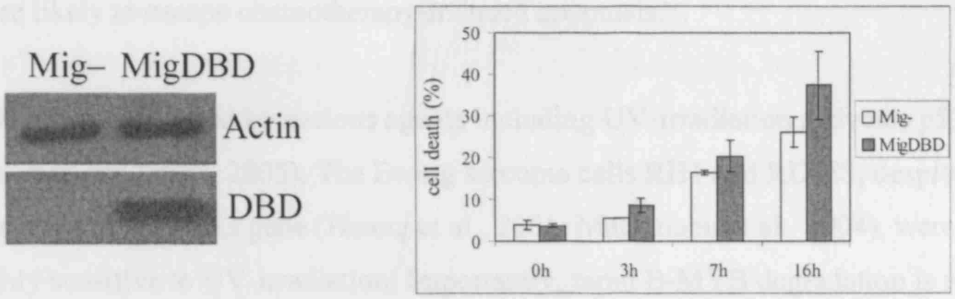


Figure 3-9 Suppressing B-MYB transactivation activity slightly sensitises neuroblastoma cells to UV-induced cell death

(A) SH-SY5Y cells were infected with retroviruses containing the B-MYB DBD or empty vector. Expression of the truncated protein was detected by western blotting with an antibody recognising the N-terminal portion of B-MYB. (B) Cell death was scored by propidium iodide DNA-staining and FACS analysis and the histogram represents the percentages of cell death observed in cells expressing the B-MYB-DBD compared to control cells at different times after UV-irradiation.

3.2.7 Discussion

DNA damaging agents can produce a wide range of DNA lesions, such as base damage, interstrand/intrastrand cross-linking as well as single and double strand breaks. UV light is electromagnetic irradiation emitted from the sun and UV-irradiation triggers oxidative events as well as induces cross-linking of adjacent DNA bases. One of the most frequent lesions induced by UV-irradiation is the cyclobutane-pyrimidine dimer (CPB), which consists of the covalent linking of two adjacent pyrimidine bases within the DNA double helix (Latonen and Laiho, 2005). While the effects of UV-irradiation are particularly relevant for skin cancers, cells derived from the neuroectoderm do not come into contact with UV light in their physiological environment. However, chemotherapeutic agents, such as cisplatin and melphalan cross-link adjacent DNA bases, or others including doxorubicin and etoposide induce oxidative stress. These chemotherapeutic drugs are commonly used to treat neuroblastoma patients (De Bernardi et al., 2003). Thus, UV-irradiation serves as a general paradigm to study DNA damage-induced effects on

cell survival and our results imply that tumours with stabilised B-MYB might be more likely to escape chemotherapy-induced apoptosis.

DNA damage elicited by various agents including UV-irradiation activates p53 (Latonen and Laiho, 2005). The Ewing sarcoma cells RH1 and RD-ES, despite carrying a mutant p53 gene (Huang et al., 2001; Matsunobu et al., 2004), were highly sensitive to UV-irradiation. Importantly, rapid B-MYB degradation is not a simple consequence of apoptosis in these cells as B-MYB degradation was not inhibited upon repression of apoptosis. In contrast, the neuroblastoma SH-SY5Y cells have an intact p53 gene and SK-N-AS cells harbour a p53 isoform that does not activate p53 target genes as efficiently as the wild type (Goldschneider et al., 2006). These neuroblastoma cells displayed only a minimal apoptotic response after UV-irradiation, which coincides with highly stable B-MYB protein. This led us to hypothesise whether increased levels of B-MYB could contribute to the apoptosis resistant phenotype of neuroblastoma cells in response to UV-induced DNA damage ultimately favouring tumour cell growth. Indeed, chicken cells with a disrupted B-MYB locus were more sensitive to UV-induced apoptosis (Ahlbory et al., 2005). As there were no means to stabilise B-MYB in Ewing sarcoma cells we studied the effect of B-MYB in response to UV-irradiation by protein overexpression. Still, a great proportion of Ewing sarcoma cells underwent apoptosis when B-MYB was overexpressed but, compared to empty vector transfected cells, the apoptotic response of the former was reduced within an early time point after UV-irradiation. The loss of protection at a later time point may be due to degradation of exogenous B-MYB by UV-irradiation.

Interestingly, B-MYB overexpression has different effects depending on the cell type investigated. Even though it is not clear why this is the case, it is thought that cell-specific expression of B-MYB binding partners allows formation of different functional complexes (Tashiro et al., 1995). Ectopic expression of B-MYB enabled glioblastoma and osteosarcoma cell lines to overcome a p107- or p53-mediated G1 checkpoint arrest by promoting entry into S phase (Lin et al., 1994; Sala et al., 1996a) and also overrode an IL6-imposed cell cycle arrest in leukaemia cells (Bies et al., 1996). In addition, enhanced B-MYB expression reduced the dependence for mitogenic signals of some cells. Constitutive B-MYB expression in IL2 dependent

murine lymphocytes resulted in diminished cytokine dependence (Grassilli et al., 1999) and reduced growth factor requirement of BALB/c 3T3 cells as well as promoted their growth in soft agar (Sala and Calabretta, 1992). Moreover, as B-MYB downregulation correlates with terminal differentiation of various cell types, B-MYB overexpression prevented growth arrest associated with terminal differentiation of monocytic cells (Bies et al., 1996) and neuroblastoma cells (Raschella et al., 1995). Importantly, constitutive B-MYB expression in lymphocytes and neuroblastoma cells resulted in resistance to chemotherapeutic agents such as doxorubicin and dexamethasone (Grassilli et al., 1999; Cervellera et al., 2000).

Consistent with these results, inhibition of B-MYB target gene induction with a B-MYB dominant negative construct diminished the resistance of neuroblastoma cells to UV-induced apoptosis to some degree. Surprisingly, neuroblastoma cells infected with the DBD of B-MYB did not exhibit decreased proliferation or increased apoptosis *per se*. This does not seem to be a prerequisite of neuroblastoma cells as unstressed fibroblasts infected with the same construct were not affected either (Santilli et al., 2005) implying that under normal culturing conditions B-MYB transcription is not required but seems to play an important role after stress.

The maintenance of genomic integrity is crucial for cells as cellular functions can be distorted when DNA is damaged and therefore poses a serious threat. Lesions in genomic DNA can result in the introduction of mutations that lead to abnormal cellular functions, cell death and genomic instability. Cells have evolved complex mechanisms to recognise DNA damage, activate cell cycle arrest and trigger repair mechanisms (Jackson, 2001). In metazoans, DNA damage is also a precursor of cancer (Elledge, 1996). Consequently, DNA damage-induced apoptosis is thought to be a physiological response aimed at eliminating potentially transforming genomic lesions. Reduced apoptosis can therefore increase tumour progression by allowing the selection of aggressive clones emerging from a pool of cells with a highly unstable genome (Latonen and Laiho, 2005). As B-MYB appears to regulate the apoptotic response to DNA damaging agents, it is likely that deregulated expression of B-MYB might be one factor facilitating cell transformation.

Overall, our results show that prolonged B-MYB expression coincides with a low apoptotic response to UV-irradiation in neuroblastoma cells. The protective effect of increased B-MYB expression levels observed with a B-MYB overexpression approach and by employing a dominant negative form of B-MYB suggests that, indeed, increased B-MYB stability might award these cells with growth advantage in stress situations that could ultimately facilitate transformation.

3.3 Mechanisms of B-MYB stabilisation in neuroblastoma cells

3.3.1 Background

Multiple levels of regulation ensure tight control over the activity of key transcription factors. The major levels of regulation include transcriptional as well as post-translational control, whereby protein degradation is a crucial post-translational regulatory event. Proteolysis is essential for many cellular functions, such as temporal degradation of proteins involved in cell cycle control, (de)activation of transcription factors and removal of incorrectly folded or damaged proteins. Protein degradation is therefore an important regulator of physiological processes and central for cell survival (Ciechanover, 1998; Laney and Hochstrasser, 1999).

Many transcription factors, which play crucial roles in embryonic development and disease, such as the Smads (Izzi and Attisano, 2004) are known to be regulated at the level of protein turnover. The 26S proteasome machinery is the main pathway regulating protein turnover in a specific manner, thereby controlling the time span during which a protein is present in a cell. Prior to proteasome-mediated degradation, ubiquitin peptides are covalently linked to destined proteins. This process is a necessary prerequisite for proteasomal degradation, whereby ubiquitination is a regulated event, governed through the action of E1, ubiquitin activating enzymes, over E2, ubiquitin transfer enzymes, to E3, ubiquitin ligating enzymes. Substrate specificity is mediated via the E3 ligases, which are specific for particular sets of proteins. The covalently linked ubiquitin residue is itself ligated to another ubiquitin molecule. This process can be subsequently repeated several times, resulting in a multi-ubiquitinated protein. Ultimately, multi-ubiquitination marks proteins for degradation (Ciechanover, 1998).

B-MYB has been shown to be a target of the proteasome degradation pathway and it is well established that phosphorylation is an important post-translational modification required to mark B-MYB for proteasomal degradation. B-MYB phosphorylation leads to subsequent addition of ubiquitin molecules through the action of the B-MYB specific ubiquitin ligase component $p45^{Skp2}$ (Charrasse et al.,

2000). Taken together these findings and our result that shows inhibition of B-MYB degradation in the presence of a proteasome inhibitor imply that the proteasome degradation pathway is presumably involved in enhanced B-MYB stabilisation in neuroblastoma cells.

There are two most likely explanations why B-MYB protein levels are more stable in neuroblastoma than in other cells. Firstly, aberrant B-MYB stabilisation could be caused by abnormal function of the degradation machinery that controls B-MYB turnover. This is that one or several factors specific for neuroblastoma cells prevent B-MYB from being degraded. Secondly, it is possible that the B-MYB gene harbours sequence alterations in neuroblastoma cells that renders it more resistant to proteasomal degradation. Thus, we set out to investigate the different possibilities.

3.3.2 Proteasomal degradation of B-MYB

Protein turnover is regulated by ubiquitinating enzymes that mark substrates for proteasomal degradation. It has been reported that B-MYB is subjected to proteasomal degradation when phosphorylated by Cdk2 and ubiquitinated by a complex containing the cell cycle-dependent ubiquitin ligase component $p45^{SKP2}$ (Charrasse et al., 2000). To assess the relevance of proteasome degradation of endogenous B-MYB turnover, a number of human cell lines were exposed to the proteasome inhibitor MG-132. Cells were harvested at different times and lysates were subjected to western blot analysis. B-MYB protein levels were monitored with an antibody specific for B-MYB (Figure 3-10). B-MYB expression increased upon proteasome inhibition in Ewing RH1 cells in a time-dependent fashion, but not in the neuroblastoma cells SH-SY5Y and SK-N-AS, demonstrating that the B-MYB degradation pathway is impaired in neuroblastoma cells.

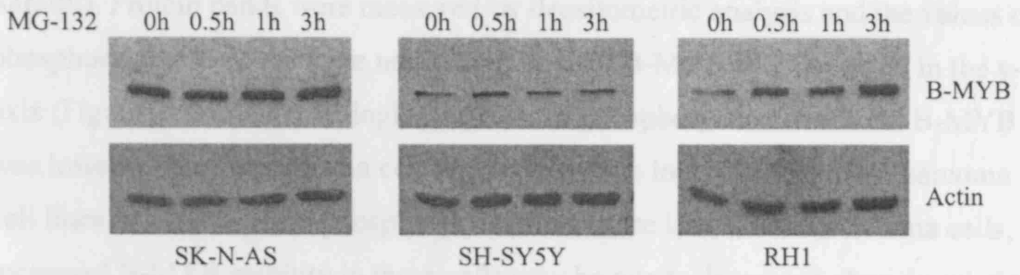


Figure 3-10 B-MYB is stabilised by the proteasome inhibitor MG-132 in Ewing sarcoma but not in neuroblastoma cell lines

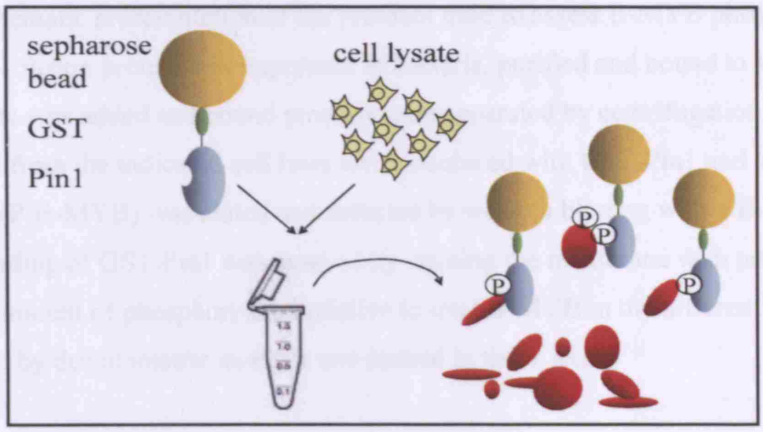
Neuroblastoma and Ewing sarcoma cell lines were grown in the presence of the proteasome inhibitor MG-132 for different periods of time. Cells were lysed and protein extracts were separated by electrophoresis. B-MYB was detected with a monoclonal antibody specifically recognising B-MYB.

3.3.3 B-MYB phosphorylation

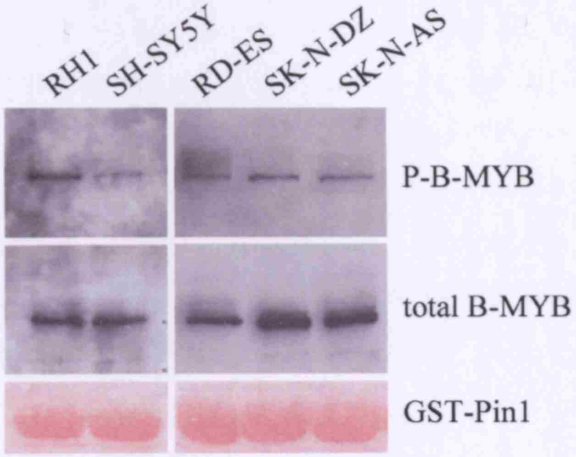
So far, we found that in neuroblastoma cells there is a defect in the B-MYB degradation pathway, but it is unclear at which step B-MYB degradation is malfunctioning. Because it is well known that B-MYB ubiquitination is dependent on phosphorylation of critical serine and threonine residues in the central and carboxy-terminus domains (Johnson et al., 2002), lack of phosphorylation could increase B-MYB half life by inhibiting its ubiquitination. This would be in agreement with the observation that MG-132 did not induce B-MYB accumulation in neuroblastoma cells (Figure 3-10). To quantify endogenous B-MYB phosphorylation we made use of a GST-fusion protein containing Pin-1. Pin-1 is a prolyl isomerase that binds to phosphorylated serine or threonine residues near to a proline (consensus sequence: P-Serine/Threonine,Proline) and regulates mitotic progression (Yaffe et al., 1997). B-MYB contains several phosphorylation sites corresponding to this motif, which are typically recognised by the Cdk2 kinase (Johnson et al., 2002). Hence, protein lysates of various cell lines were incubated with GST-Pin-1. Bound proteins were purified by centrifugation and subjected to western blot analysis with a B-MYB antibody (Figure 3-11A). B-MYB binding was abolished when the Pin-1 binding site was mutated, demonstrating that the

interaction between B-MYB and Pin-1 is specific (personal communication Dr. G. Santilli). Protein bands were measured by densitometric analysis and the values of phosphorylated B-MYB were normalised to total B-MYB and displayed in the y-axis (Figure 3-11B). Interestingly, the ratio of phosphorylated over total B-MYB was lower in 3 neuroblastoma cell lines than in two independent Ewing sarcoma cell lines. As the B-MYB phosphorylation levels are low in neuroblastoma cells, increased B-MYB stability in these cells may be due to decreased phosphorylation and presumably lack of subsequent proteasomal degradation.

A



B



C

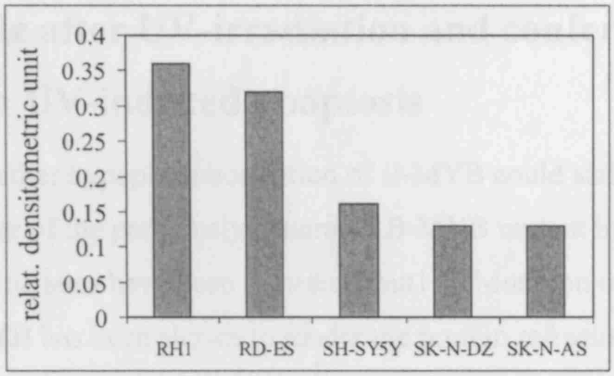


Figure 3-11 B-MYB is hypophosphorylated in neuroblastoma cell lines

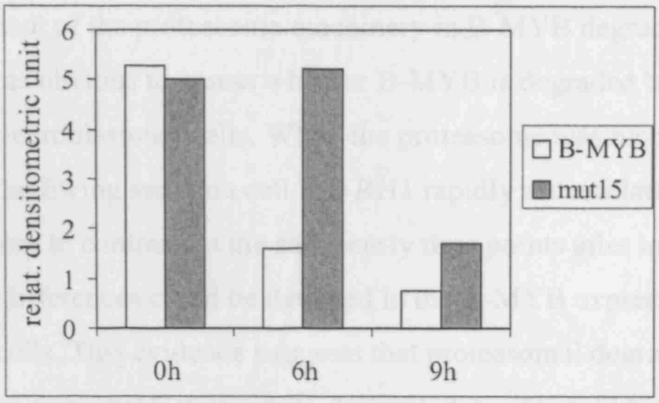
(A) A schematic representation of the protocol used to assess B-MYB phosphorylation. GST-Pin1 fusion protein was expressed in bacteria, purified and bound to sepharose beads. Cell lysate was added and bound proteins were separated by centrifugation. (B) Proteins extracted from the indicated cell lines were incubated with GST-Pin1 and bound phospho-B-MYB (P-B-MYB) was eluted and detected by western blotting with a B-MYB antibody. Equal loading of GST-Pin1 was assayed by staining the membrane with ponceau S solution (C) The amount of phosphorylated relative to total B-MYB in the different cell lines was evaluated by densitometric analysis and plotted in the Y axis.

3.3.4 Phosphorylation deficient B-MYB protein is more stable after UV-irradiation and confers protection from UV-induced apoptosis

To assess whether hypophosphorylation of B-MYB could stabilise the protein we took advantage of the previously generated B-MYB mutant in which all Cdk2 phosphorylation sites have been mutated (mut15). Mutation of 15 phosphorylation sites of B-MYB has been shown to render the protein refractory to phosphorylation and has been shown to cause inactivation of its transactivating ability on the FGF-4 promoter (Johnson et al., 2002).

Exogenous B-MYB can be readily detected in 293 cells with an antibody specific against the Flag epitope due to efficient transfection in these cells. Therefore, a control plasmid, Flag-tagged wild type or mut15 B-MYB were transfected into 293 cells, which were then subsequently UV-irradiated. Protein lysates were prepared at the indicated times and subjected to western blot analysis with an antibody specifically recognising Flag (Figure 3-12A). Similarly to what we observed in RH1 cells, wild type B-MYB is rapidly destroyed in 293 cells following UV-irradiation. In contrast, mutations of the Cdk2 phosphorylation sites render B-MYB more stable in this context, formally demonstrating that phosphorylation is a prerequisite for B-MYB degradation post UV-irradiation. As the phosphorylation deficient mutant of B-MYB has been reported to be transcriptionally deficient, one would assume that therefore it would not show any protective effect from UV-irradiation induced apoptosis. Consequently, the transfected 293 cells were harvested at the indicated time point after UV-irradiation and cell death was measured by propidium iodide staining and FACS analysis (Figure 3-12B). Surprisingly, the B-MYB mutant displayed a similar effect than wild type B-MYB in protecting 293 cells from UV-induced apoptosis. This clearly demonstrates that B-MYB phosphorylation is neither required nor necessary for its anti-apoptotic function following UV-irradiation.

A



B

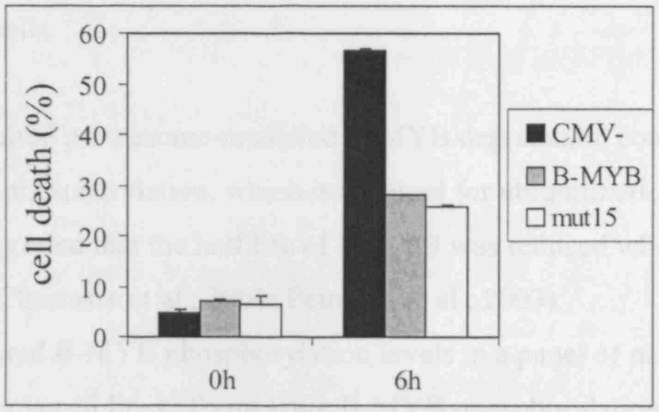


Figure 3-12 A phosphorylation-deficient B-MYB mutant is resistant to UV-induced degradation and confers protection from apoptosis

293 cells were transfected with control, wild type or mut15 B-MYB expression vectors and subjected to UV-irradiation (A). Cells were harvested at the indicated times after UV-treatment and proteins were subjected to western blot analysis with a FLAG antibody. The histogram depicts the densitometric reading obtained with B-MYB or mut15 normalised to Actin. A representative experiment is shown. (B) Cell death was monitored by propidium iodide staining and FACS analysis. The protective effect of wild type (Student's t-Test, $p=0.029$; $n=6$) and mutant B-MYB is statistically significant (Student's t-Test, $p=0.024$; $n=6$) when compared to the empty plasmid control.

3.3.5 Discussion

As the involvement of the proteasome machinery in B-MYB degradation is well established, it was obvious to assess whether B-MYB is degraded by proteasomal mechanisms in neuroblastoma cells. When the proteasome was blocked, B-MYB extracted from the Ewing sarcoma cell line RH1 rapidly accumulated in a time dependent fashion. In contrast, at the same early time points after inhibition of the proteasome, no differences could be detected in the B-MYB expression levels in neuroblastoma cells. This evidence suggests that proteasomal degradation of B-MYB is impaired in neuroblastoma cells. Nevertheless, it would have been interesting to extend the time course to evaluate whether proteasomal degradation is simply delayed or indeed not involved in the destruction of B-MYB in neuroblastoma cells.

Delayed or impaired proteasome-mediated B-MYB degradation could be caused by lack of B-MYB phosphorylation, which is required for ubiquitination. Previous reports have suggested that the half life of B-MYB was reduced when cyclin A was overexpressed (Charrasse et al., 2000; Petrovas et al., 2003).
Thus, we compared B-MYB phosphorylation levels in a panel of neuroblastoma and Ewing sarcoma cell lines. To measure B-MYB phosphorylation in a quantitative manner we used the peptidyl-prolyl isomerase Pin-1 fused to GST. Pin-1 specifically binds to motifs consisting of serines/threonines followed by a proline only when either the serine or the threonine is phosphorylated (Yaffe et al., 1997). This approach has previously been used to assess protein phosphorylation (He et al., 2003; Ley et al., 2004). Notably, Ewing sarcoma cell lines showed a greater extent of B-MYB phosphorylation compared to the phosphorylation levels of B-MYB in neuroblastoma cell lines, corroborating the theory that hypophosphorylation contributes to increased B-MYB stability. These results also suggest that the phosphorylation level of B-MYB is dependent on the cellular context.

As low levels of B-MYB phosphorylation correlate with increased B-MYB stability in neuroblastoma cell lines, it is likely that impaired phosphorylation of B-MYB in

these cells prevents B-MYB ubiquitination as well as subsequent degradation in response to DNA damage.
To address this possibility we used the B-MYB mut15 construct, where all known B-MYB phosphorylation sites were mutated (Johnson et al., 2002). Indeed, comparing ectopic expression levels of normal, B-MYB with B-MYB mut15 after UV-irradiation revealed that the half life of phosphorylation deficient B-MYB was greatly enhanced.

Various scenarios could be envisaged that may contribute to hypophosphorylation-induced stabilisation of B-MYB in neuroblastoma cells. One possibility is that protein complexes required for phosphorylation of B-MYB, such as the cyclin A/Cdk2 complex are defective in exerting their function in neuroblastoma cells. However, general absence of Cdk2 activity is an unlikely scenario, since it has been documented that the important Cdk inhibitor $p21^{Waf1/Cip1}$ is non-functional in neuroblastoma cells and that Cdk2 kinase activity and histone H2 phosphorylation can be strongly induced by DNA-damage in neuroblastoma SH-SY5Y cells, which have been extensively used in this study (Yu et al., 2005).
Abnormal protein expression is a hallmark of many cancers including neuroblastoma (Brodeur et al., 1997). Aberrant protein expression could result in complex formation with B-MYB and thus shielding B-MYB from becoming accessible by the Cdk2/cyclin A complex. Indeed, several proteins have been shown to physically interact with B-MYB and additionally influence its transactivation potential (Sala et al., 1996b; De Falco et al., 2000; Horstmann et al., 2000b; Li and McDonnell, 2002; Pilkinton et al., 2006).
Another possibility is that the cause of reduced B-MYB phosphorylation in neuroblastoma cells rests in the increased activity of a yet to be discovered B-MYB specific phosphatase regulatory subunit. The mammalian protein PER2 is an important regulator of the circadian rhythm and its degradation is regulated through a similar mechanism as the one proposed for B-MYB. While PER2 phosphorylation targets the protein for proteasomal degradation, Gallego et al. have demonstrated that protein phosphatase 1 increases the stability of PER2 (Gallego et al., 2006). Overexpression of phosphatases would presumably prove the principle that hypophosphorylation of B-MYB contributes to its stabilisation, but this approach

could hardly be used to further study the consequences of increased B-MYB stability due to the many cellular substrates of a phosphatase (Bollen, 2001). Apart from phosphorylation, other post-translational modifications could contribute to the regulation of B-MYB degradation. For example, proteasomal degradation of Smad7 is prevented by the acetylation function of p300. Acetylation of Smad7 occurs at the same lysine residues that also serve as platforms for covalent binding of ubiquitin. Accordingly, it was proposed that competition between acetylation and ubiquitination regulates the stability of Smad7 (Gronroos et al., 2002). A similar mechanism could apply for B-MYB stabilisation in neuroblastoma cells, as B-MYB has previously been shown to be acetylated by the co-activator p300. However, the significance of B-MYB phosphorylation for acetylation remains unclear to date as one group reports that B-MYB acetylation occurs in a phosphorylation independent manner (Johnson et al., 2002) whereas another group observed that B-MYB acetylation is stimulated by B-MYB phosphorylation (Schubert et al., 2004). Sumoylation is another post-translational modification implicated in the regulation of protein stability. Even though there is no information available on whether B-MYB is subject to sumoylation, SUMO-1 was found to be covalently linked to c-MYB. This in turn stabilises c-MYB though an unknown mechanism, which is not based on competition between sumoylation and ubiquitination (Bies et al., 2002).

Saville and colleagues reported that expression levels of B-MYB were decreased by cotransfection of cyclin A or increased when B-MYB Cdk2 phosphorylation sites were mutated (Saville and Watson, 1998). This observation reinforces the hypothesis that increased B-MYB stability contributes to protein accumulation similar to a situation where gene amplification occurs. While this hypothesis suggests a role for B-MYB in tumourigenesis, the question remains whether increased levels of hypophosphorylated B-MYB could indeed interfere with cellular control mechanisms. B-MYB is known to transcriptionally regulate proteins involved in the control of cell cycle and apoptosis, but B-MYB phosphorylation is required to transactivate some target genes. Despite this fact, we show that the phosphorylation deficient mutant of B-MYB, which is transcriptionally dysfunctional on certain promoters, retains the ability to protect cells from UV-induced apoptosis. It would have been interesting though to assess whether at later time points the phosphorylation deficient B-MYB construct might even be more

capable of mediating protection from apoptosis than its phosphorylation competent counterpart and thus provide a growth advantage to phosphorylated B-MYB. Since phosphoryolation deficient B-MYB is able to protect cells from apoptosis, it is possible that B-MYB not only regulates cell survival in a transcription dependent manner, through the regulation of genes like Bcl-2, but also independently of the function as a transcription factor. This is reminiscent of the apoptotic induction by p53, which mainly occurs by directly transcribing genes required for the apoptotic pathway such as Bax, PUMA and Noxa (Latonen and Laiho, 2005), but p53 has also been shown to activate caspase-8 in a transcription independent fashion (Ding et al., 2000).

As the phosphorylation deficient mutant of B-MYB has been reported to be transcriptionally deficient on certain promoters, one might assume that it would therefore not show any biological activity. However, evidence already exists indicating that B-MYB is able to exert certain cellular functions, such as promoting cell proliferation by non-transcriptional mechanisms. Ectopically expressed B-MYB is able to circumvent a p107-induced growth arrest and this effect has been proposed to be due to direct physical interaction between B-MYB and p107. The B-MYB C-terminus is required for B-MYB interaction with p107 and B-MYB phosphorylation was not found to contribute to the interaction (Joaquin et al., 2002). B-MYB also partially overcomes a $p57^{KIP2}$-induced cell cycle arrest whereby the N-terminal domain of B-MYB is necessary (Joaquin and Watson, 2003b).

Another paradox arises when comparing the effect on cell survival by either studying the overexpression of the B-MYB DBD or the phosphorylation deficient mutant of B-MYB. The DBD is thought to compete with endogenous B-MYB for DNA binding sites and thus inhibits transcription of B-MYB target genes, which in fact has been show for ApoJ/clusterin (Santilli et al., 2005). While overexpression of dominant negative B-MYB conferred sensitivity towards UV-induced apoptosis, phosphorylation deficient B-MYB awarded protection from UV-induced apoptosis. An explanation for this discrepancy might lie in the dual role of B-MYB whereby B-MYB can govern cell function via transcription dependent as well as independent mechanisms as mentioned above, whereby the N-terminal domain of B-MYB is required for at least some transcription-independent functions.

Another reason might be that B-MYB is able to transactivate promoters in a binding site dependent manner as well as in a DNA binding independent fashion. In the latter case B-MYB acts as a transcription co-factor via interactions with other transcription factors. Generally, for MBS dependent transactivation, the B-MYB transactivation potential is affected positively by cyclin A/Cdk2 dependent phosphorylation (Robinson et al., 1996; Lane et al., 1997; Sala et al., 1997; Bartsch et al., 1999). B-MYB phosphorylation is thought to be required for transcriptional activation to alleviate an inhibitory effect mediated by the C-terminal domain of B-MYB. Importantly, transactivation of MBS-independent promoters seems to function in the absence of B-MYB phosphorylation (Kamano and Klempnauer, 1997; Sala et al., 1999; Bartusel et al., 2005). Especially on promoters where B-MYB cooperates with Sp1 for transactivation, B-MYB is not subjected to the inhibitory effect of the C-terminal domain, but actually requires this domain (Bartusel et al., 2005). Thus, while overexpression of the B-MYB DBD or a phosphorylation deficient B-MYB block direct transactivation of pro-survival genes, phosphorylation deficient B-MYB is presumably still able to transactivate other potential pro-survival genes that require B-MYB as a co-factor.
It is possible that the activation of MBS-independent promoters by B-MYB is part of a damage-response mechanism. To take this hypothesis even further, it might be possible that hypophosphorylated B-MYB is even more efficient in cooperating with Sp1 or other transcription factors because in this context, the B-MYB C-terminus, which harbours virtually all phosphorylation sites might not confer an inhibitory effect (Bartusel et al., 2005). Moreover, B-MYB phosphorylation could abolish protein interactions that allow B-MYB to cooperate with other transcription factors on MBS-independent promoters. However, this theory awaits further investigation.

An elegant way to further elucidate the effect of slowly degrading B-MYB would be the use of a B-MYB specific phosphatase holoenzyme. Such an enzyme complex could be overexpressed in cells with rapidly degrading B-MYB, ideally in primary, sympathetic neurons that have not yet accomplished terminal differentiation. The use of such a specific phosphatase might presumably stabilise B-MYB and thus confirm or reject the theory that increased B-MYB stabilisation confers a protective effect on the cells upon UV-induced DNA damage. Nevertheless such a B-MYB

specific phosphatase complex has not been identified to date and aggravates the attempt to further test our observations for general applicability.

Overall, it would be interesting to understand the molecular mechanism in detail that causes decreased B-MYB phosphorylation in neuroblastoma cells. Unravelling this mechanism could open new avenues to target abnormally stabilised B-MYB protein levels and potentially halt uncontrolled proliferation and/or induce apoptosis in neuroblastoma cells.

3.4 Analysis of B-MYB mutations in neuroblastoma cells

3.4.1 Background

We have established that following UV-irradiation B-MYB protein can be stabilised, to some extent, when B-MYB phosphorylation is restrained. Furthermore, low endogenous B-MYB phosphorylation levels in neuroblastoma cells strongly suggest that decreased B-MYB phosphorylation contributes to B-MYB protein stabilisation in these cells. However, the half life of endogenous B-MYB in several neuroblastoma cell lines is prolonged far beyond that of the ectopically expressed phosphorylation deficient B-MYB mutant following UV-treatment. This evidence suggests that additional factors might contribute to stabilise B-MYB in neuroblastoma cells.

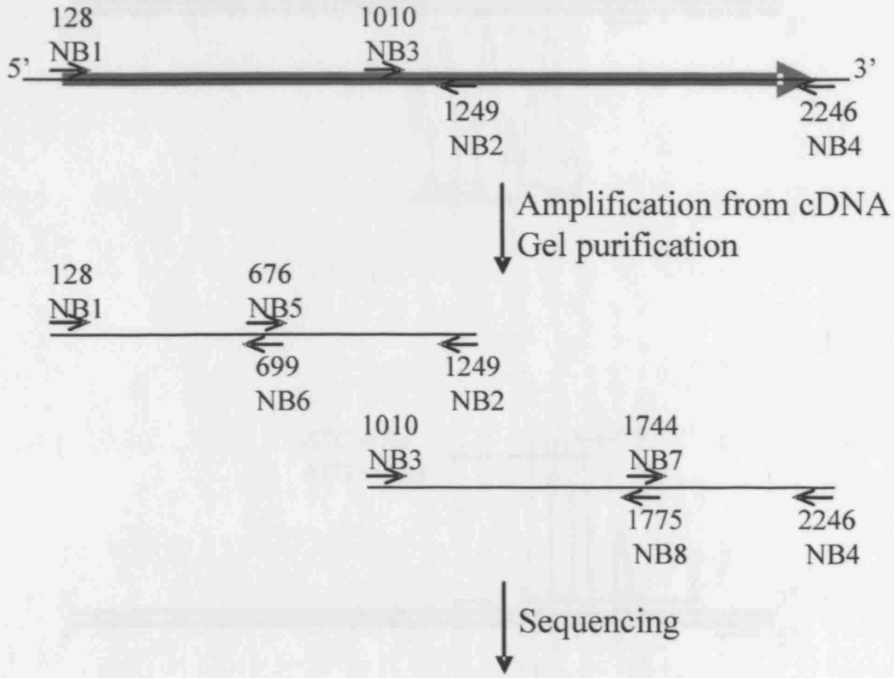
Mutations are a common cause of increased protein stability. For example, C-terminally truncated c-MYB in leukaemic cell lines was found to be more stable than full-length or N-terminally truncated c-MYB. It appears, that the loss of as little as 80 C-terminal amino acids renders c-MYB more resistant to proteasome-mediated destruction (Bies and Wolff, 1997). Interestingly, cells expressing C-terminally truncated c-MYB showed higher transformation ability than cells expressing full-length c-MYB (Ferrao et al., 1995). These findings have prompted us to analyse the coding sequence of B-MYB for sequence variations. Comparing the sequencing results of endogenous B-MYB from several neuroblastoma cell lines with that of the published B-MYB sequence, which is from now on referred to as the wild type sequence, allows detecting neuroblastoma-specific B-MYB sequence alterations, which could potentially enhance B-MYB stability.

3.4.2 B-MYB sequencing

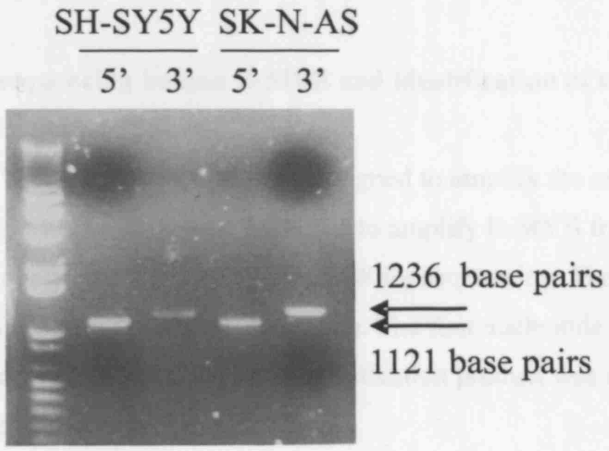
To test whether sequence alterations occur in the coding region of B-MYB in neuroblastoma cells, total RNA was extracted from the two neuroblastoma cell lines SK-N-AS and SH-SY5Y and reverse transcribed. B-MYB cDNA was PCR-amplified in a 5'- and a 3'-portion. The two DNA fragments were gel purified and

sequenced (Figure 3-13A, B). An overview of the sequencing strategy is shown in Figure 3-13A.

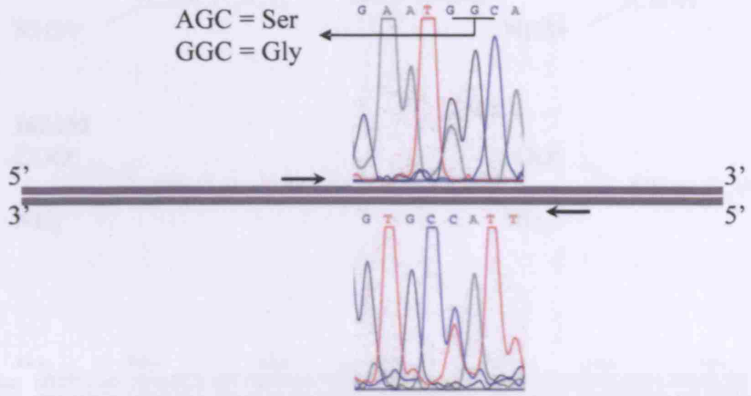
A



B



C



D

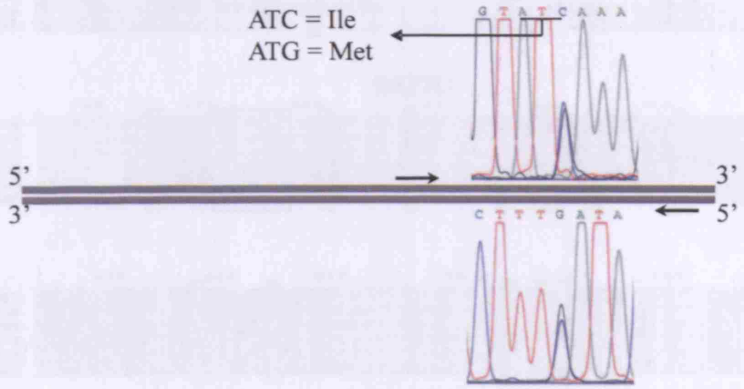
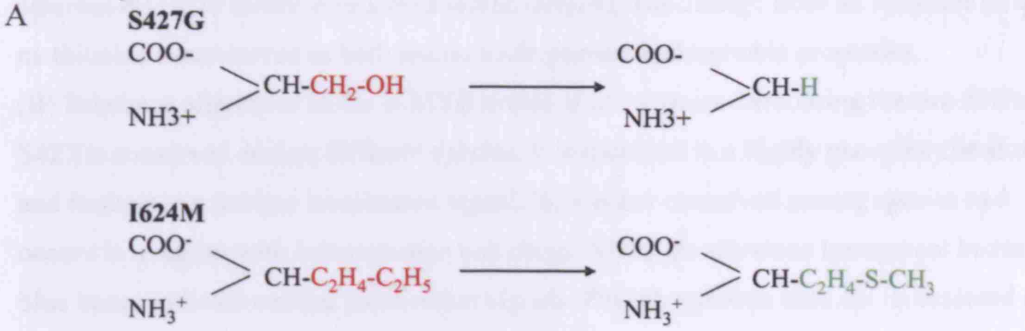


Figure 3-13 Strategy for sequencing human B-MYB and identification of two polymorphisms in neuroblastoma cell lines

(A) PCR primer pairs NB1, NB2 and NB3, NB4 were designed to amplify the entire coding sequence of B-MYB in two portions. They were first used to amplify B-MYB from cDNA, and then in conjunction with primers NB5, NB6, NB7, NB8 for sequencing. The number above a primer indicates the position of the first nucleotide. The first nucleotide of the ATG start codon is at position 128. (B) The B-MYB amplification product was separated on an agarose gel and purified from the gel.

Chromatograms of sense- and antisense sequencing results. (C) SK-N-AS cells are heterozygous for the triplet GGC instead of AGC. This triplet is at codon position 427 from the ATG translation initiation codon. (D) SH-SY5Y cells were found to be heterozygous in codon number 624. The major codon is ATC, which is changed to ATG in SH-SY5Y cells.

A



B

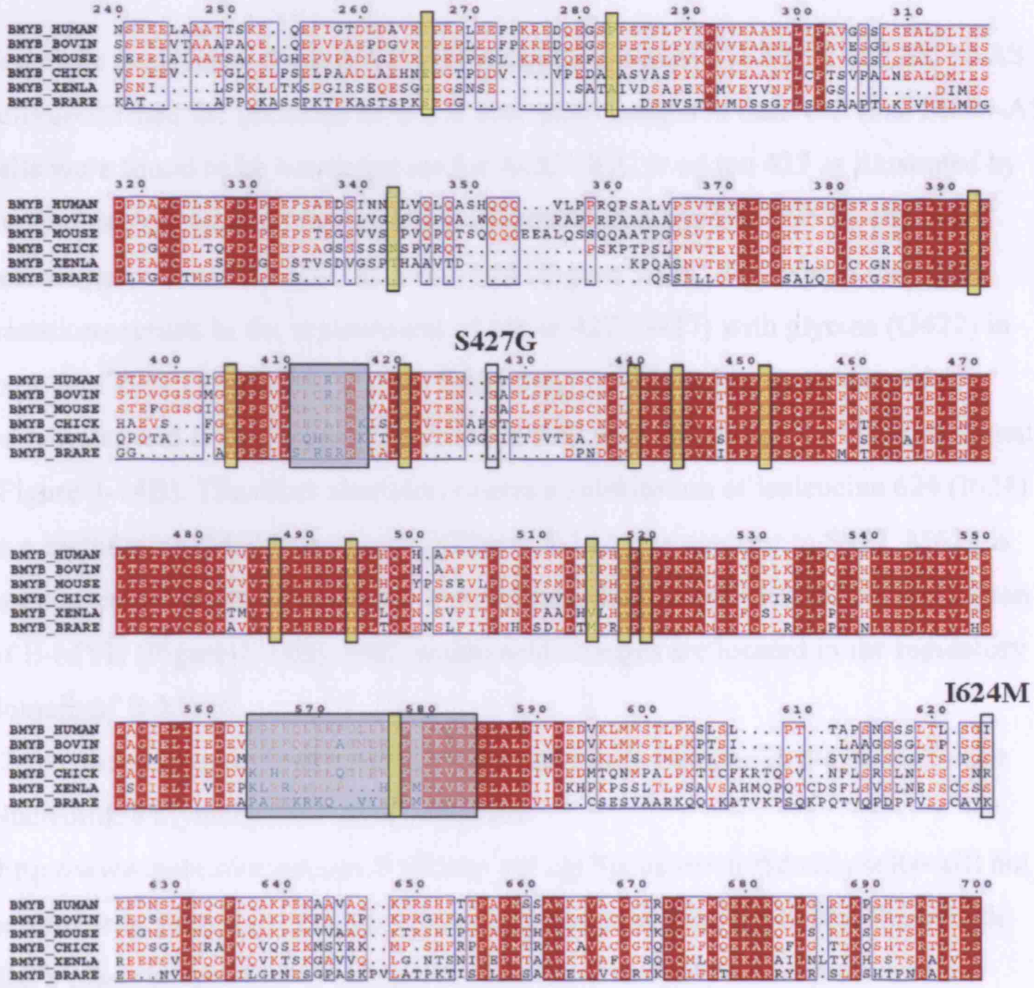


Figure 3-14 The identified SNPs are non-synonymous

(A) Triplet GGC427 encodes a glycine (G427) and thus replaces a serine that is encoded by the major codon. This is a considerable change as glycine does not have a side chain and is the only non-chiral amino acid. The major triplet at position 627 encodes an isolycine

whereas ATG624 determines a methionine (M624). The change from an isolycine to a methionine is conserved as both amino acids possess hydrophobic properties.
(B) Sequence alignment of the B-MYB amino acid sequence harbouring the two SNPs. S427 is conserved among different species. It is localised in a highly phosphorylated region and is close to a nuclear localisation signal. I624 is not conserved among species and occurs in a region with low sequence homology. SNPs are shown as transparent boxes and blue boxes indicate nuclear localisation signals. Phosphorylation sites are represented as yellow boxes and their postitions correspond to the phosphorylation sites in murine B-MYB, but these sites are conserved in human B-MYB.

Sequence analysis of the B-MYB cDNAs obtained from SH-SY5Y and SK-N-AS cells confirmed the presence of single base pair changes in each cell line. SK-N-AS cells were found to be heterozygous for AGC/GGC at codon 427 as illustrated by the double A/G peak on the sequence chromatogram and SH-SY5Y cells are heterozygous for ATC/ATG at codon 624 (Figure 3-13C, D). One of the point mutations results in the replacement of serine 427 (S427) with glycine (G427) in exon 8 of B-MYB (Figure 3-14A). Interestingly, S427 is highly conserved in vertebrates and is localised adjacent to the first B-MYB nuclear localisation signal (Figure 3-14B). The other alteration causes a substitution of isoleucine 624 (I624) to a methionine (M624) in exon 13 (Figure 3-14A). In contrast to S427, M624 is not conserved among species and is positioned in an overall less conserved region of B-MYB (Figure 3-14B). Both amino acid changes are located in the regulatory domain of B-MYB.
These two amino acid substitutions were previously mapped in the NCBI Single Nucleotide Polymorphism (SNP) database (http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?locusId=4605&chooseRs=all) but no further information is published on their potential functional role. Since both SNPs cause an amino acid change, the structural integrity of the protein or the ability to form protein-protein interactions could be altered, potentially affecting B-MYB function.

3.4.3 Single amino acids exchanges in the B-MYB protein do not alter protein stability

No other sequence alterations, except the SNPs described above, were found in the coding region of B-MYB in two neuroblastoma cell lines that display highly stable B-MYB. Even though the sequence changes are minimal, we speculated that they might nevertheless influence protein stability. To be able to test this theory, the identified base pair substitutions were introduced into a B-MYB expression vector by site directed mutagenesis and the resulting constructs were confirmed by sequencing. A schematic drawing of the resulting B-MYB expression products is shown in Figure 3-15A. The different B-MYB constructs were transfected into 293 cells, which were subsequently UV-irradiated. Cells were then harvested at the indicated time points and lysates were subjected to western blot analysis with an antibody specifically recognising exogenous B-MYB (Figure 3-15B). As previously observed, wild type B-MYB degraded rapidly in 293 cells upon UV-treatment and the two variant forms of B-MYB turned out to be degraded with similar kinetics. This experiment clearly demonstrates that the identified B-MYB sequence alterations in neuroblastoma cells do not markedly affect protein stability.

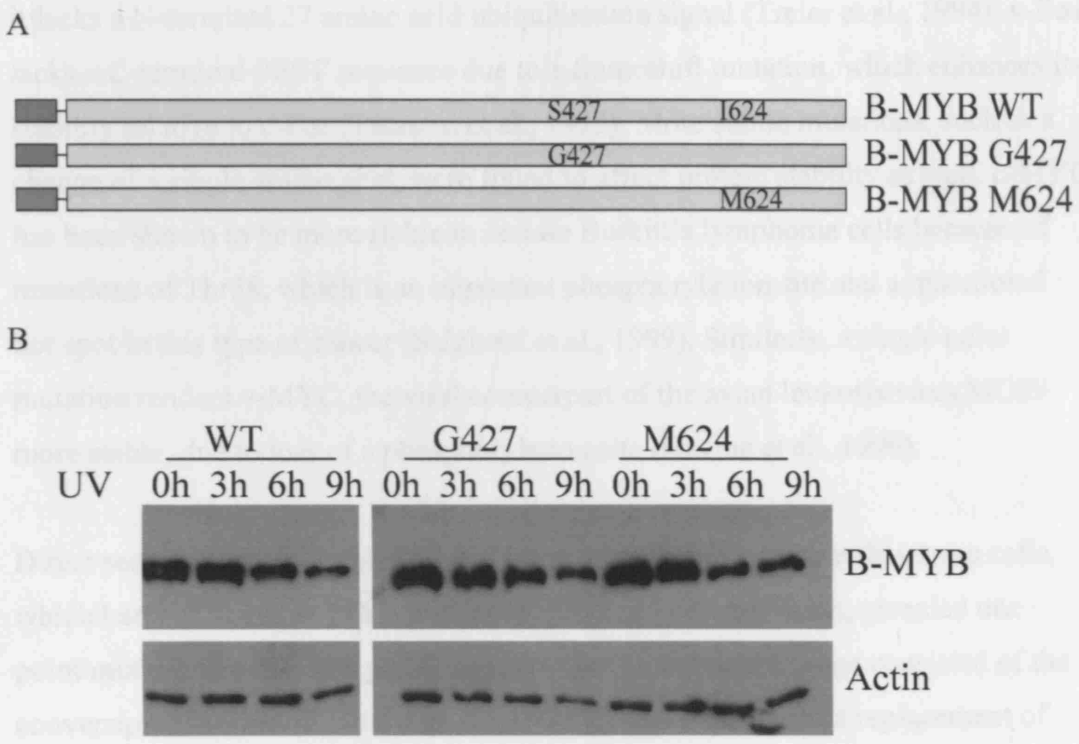


Figure 3-15 B-MYB variants do not affect protein stability

(A) Point mutations were introduced into the pcDNA3.1/HisB-MYB construct by site directed mutagenesis. Cytidine at position 1872 was replaced with a guanosine and adenosine at position1279 with a cytidine to generate the constructs pcDNA3.1/HisB-MYBG427 and pcDNA3.1/HisB-MYBM624, respectively. A schematic representation of the expression products is shown. (B) 293 cells were transfected with the B-MYB variants and subsequently UV-irradiated. Lysates were prepared at the indicated times and proteins were separated by electrophoresis. Exogenous B-MYB was detected with an Xpress antibody specifically recognising the tag.

3.4.4 Discussion

A common cause of oncogenic activation of regulatory proteins involves sequence mutations, whereby the protein may be constitutively activated or stabilised. Such mutations are particularly widespread in viral oncogenes. Viral oncogenes are normally homologous to their cellular progenitors but additionally harbour crucial sequence variations, conferring the protein with the ability to promote transformation. Treier et al. have reported that v-Jun escapes ubiquitination because

it lacks a N-terminal 27 amino acid ubiquitination signal (Treier et al., 1994). v-Fos lacks a C-terminal PEST sequence due to a frameshift mutation, which enhances its stability relative to c-Fos (Tsurumi et al., 1995). More subtle mutations, such as a change of a single amino acid, were found to affect protein stability as well. c-MYC has been shown to be more stable in certain Burkitt's lymphoma cells because of mutations of Thr58, which is an important phosphorylation site and a mutational hot spot in this type of cancer (Salghetti et al., 1999). Similarly, a single point mutation renders v-MYC, the viral counterpart of the avian leukosis virus MC29 more stable, due to loss of a phosphorylation site (Gavine et al., 1999).

Direct sequencing of the B-MYB cDNA extracted from two neuroblastoma cells, which harbour stable B-MYB protein levels after UV-irradiation, revealed one point mutation at different positions each. One amino acid change consisted of the conversion of serine 427 into a glycine (G427) and the other of a replacement of isoleucine 624 with a methionine (M624). Both alterations were found to be previously mapped SNPs. While both the SNPs are located in the B-MYB regulatory domain, particularly SNP S427G could be functionally relevant because S427 is highly conserved in all vertebrates and conserved amino acids are likely to be important for proper protein function. S427 is placed close to the first B-MYB nuclear localisation signal and is adjacent to highly conserved regions of the B-MYB regulatory domain (see amino acid sequence alignment in Figure 3-14B). The change from serine to glycine results in the loss of a hydrogen bond donor group (a hydroxyl group). This additional hydroxyl group of serine could contribute to important hydrogen bonds within B-MYB or with binding partners of B-MYB, which is consequently lost when substituted with a glycine. Furthermore, glycine is the only non-chiral amino acid and shows therefore higher conformational flexibility, namely it can adopt either a cis- or a trans-conformation. Thus, a change from a serine into a glycine could mean that the protein acquires greater structural flexibility potentially leading to a conformational change or even introduce potential disorder in the protein fold. Although S427 has not been identified as a B-MYB phosphorylation site to date, it cannot be excluded that it is a possible phosphorylation site and therefore this SNP could modulate B-MYB regulation.
As for the I624M SNP, the change from an isoleucine to methionine maintains the hydrophobic character of the amino acid but results in an increase in the size of the

side chain. Furthermore, an additional functionality is introduced with the extra sulphur group of methionine, as methionine together with cysteine and tryptophan is one of the most readily oxidised amino acids. Oxidation of the sulphur group in methionine has been implicated to result in loss as well as in activation of protein function (Vogt, 1995). Redox reactions also affect the activity of transcription factors as reduction mediated by either reducing agents or by nuclear redox factor (Ref-1) of a cysteine residue in the Jun-Fos heterodimer is required for binding of AP-1 to DNA (Abate et al., 1990).

In order to test whether the two amino acid substitutions might affect protein stability, two constructs were generated by site directed mutagenesis each encoding for one of the B-MYB SNPs. However, expression of the three different B-MYB forms and subsequent UV-irradiation revealed that neither of the minor alleles did affect protein stability with respect to wild type B-MYB. Despite point mutations were previously shown to alter protein turnover, the sequence alterations we detected in neuroblastoma cell lines do not contribute to increased B-MYB stability, but might affect other aspects of B-MYB function.

3.5 Functional analysis of B-MYB variants

3.5.1 Background

The detection of two polymorphisms in the B-MYB coding sequence was described in the previous chapter. Both alterations consist of single base pair changes and were previously mapped in the NCBI SNP database. Both base pair substitutions generate an alternative nucleotide triplet each that encodes for a different amino acid. The SNP in exon 8 results in the expression of either serine (S427) or glycine (G427) at codon 427, whereby the latter is the minor allele. In exon 13, methionine (M624) replaces the more frequently occurring isoleucine (I624) at position 624. SNPs commonly arise by insertions, deletions or point mutations involving only one nucleotide. SNPs are the most frequent alterations in the genome and they account for approximately 90% of all sequence variations in humans. Estimations assume that about one SNP occurs per 1000 bases (Wang and Moult, 2001). Thus, every gene contains one or two SNPs in average, whereby only about half of the SNPs alter the resulting amino acids (Hemminki and Shields, 2002). Some authors merely refer to the term SNP if the variant allele occurs with at least 1% frequency in the population (Engle et al., 2006).

While not all non-synonymous SNPs display an altered phenotype, there is currently a rapidly growing body of evidence indicating that some SNPs can affect protein function and thus are able to modulate the susceptibility to disease or the response to drugs. SNPs might influence protein function by altering protein expression, stability, folding, ligand binding, catalysis, signalling and post-translational modifications (Wang and Moult, 2001).

In this chapter I aim to characterise both the novel variants of B-MYB by comparing various aspects of B-MYB regulation and function to wild type B-MYB.

3.5.2 B-MYB variants protect cells less efficiently from H_2O_2-induced cell death

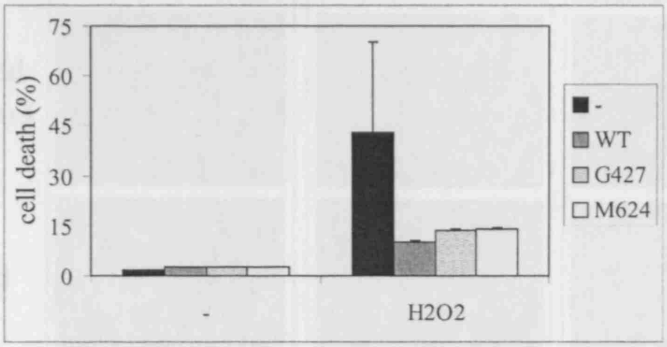
We were interested what biological effect would result from the identified single point mutations in the coding region of B-MYB. Since B-MYB is known to modulate the cell's response to different stress stimuli (Grassilli et al., 1999; Cervellera et al., 2000), we assessed the effect of B-MYB overexpression in response to H_2O_2. H_2O_2 belongs to the family of reactive oxygen species (ROS), which are products of normal cell metabolism. Overproduction of ROS results in oxidative stress and has been linked to different diseases, such as cancer, Alzheimer's disease as well as Parkinson's disease and conditions, including ischemia (Valko et al., 2007).

293 cells, transfected with the different B-MYB expression constructs and an empty vector as control, were treated with 50 μM H_2O_2. After culturing the cells for 24 hours they were harvested and stained with propidium iodide. Cell cycle profiles were assessed by FACS analysis (Figure 3-16A) and B-MYB expression levels were analysed by western blot with an antibody specific for ectopic B-MYB (Figure 3-16B). All three forms of B-MYB clearly protected 293 cells from H_2O_2-induced apoptosis. In three independent experiments the B-MYB variants were slightly but significantly less efficient in protecting cells from H_2O_2-induced cell death.

Next, we thought to also evaluate our data on cell death on the single cell level. Therefore, we transfected 293 cells with the three B-MYB constructs and subsequently cultured them on coverslips. Cells were then fixed with para-formaldehyde and exogenous B-MYB was labelled in green with the Xpress antibody. Activated caspase-3 was stained in red with an antibody specifically recognising the activated form of caspase-3 and nuclei were visualised with DAPI staining. Ectopic B-MYB expressing cells were scored and visually assessed whether they were positive for activated caspase-3 (Figure 3-17). B-MYB positive cells were counted and the number of activated caspase-3 positive cells is displayed as a ratio of the total number of counted cells (Table 3-1). The wild type form of B-MYB confers greater protection from H_2O_2-induced cell death than the G427 and

M624 variants. Even though the differences were moderate, they were nevertheless reproducible and statistically significant.

A



B

Figure 3-16 The different forms of B-MYB confer protection from H_2O_2-induced cell death

(A) 293 cells were transfected with the B-MYB constructs and cells were incubated with 50 μM H_2O_2 for 24 hours. Cells were harvested, fixed in ethanol and stained with propidium iodide. Cell death was assessed by FACS analysis and a representative experiment is shown. Both, the differences of cell death between cells transfected with wild type B-MYB or the G427 variant (Student's t-test, p= 0.033, n=6) and between wild type B-MYB or the M624 variant (Student's t-test, p=0.027, n=6) were statistically significant. (B) Protein expression was assessed by western blot with an antibody detecting tagged B-MYB.

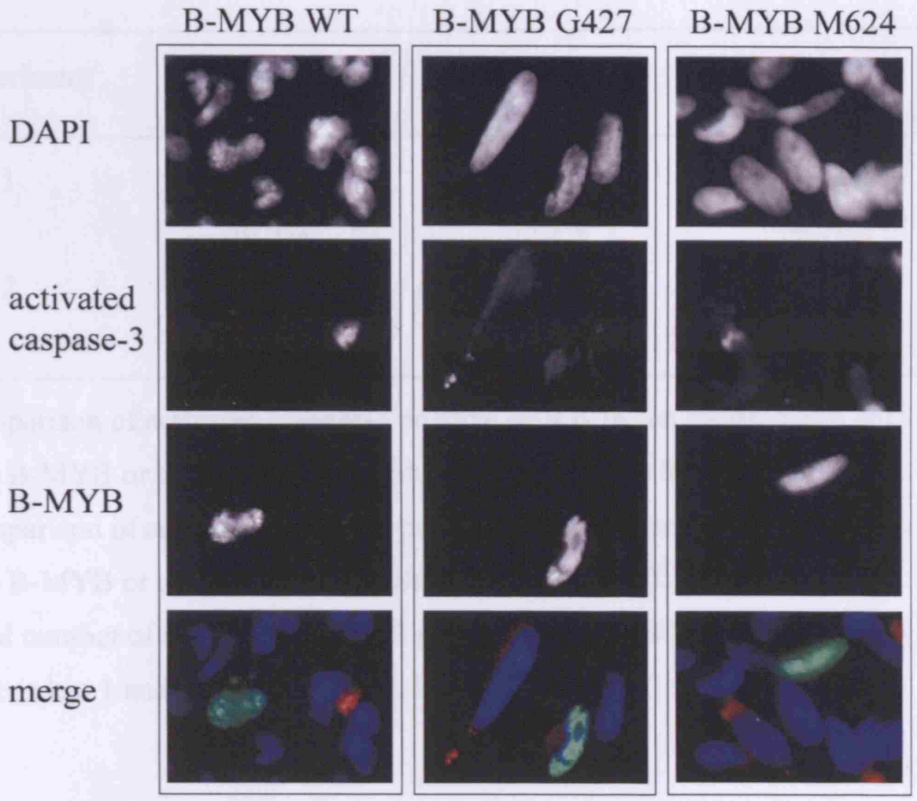


Figure 3-17 The B-MYB variants protect cells less efficiently from H_2O_2-induced cell death

293 cells were transfected with the B-MYB constructs, grown on cover slips and treated with 50 µM H_2O_2. 24 hours after H_2O_2 treatment, cells were fixed with paraformaldehyde and subsequently stained with antibodies cross-reacting with activated caspase-3 (red) and the Xpress epitope on ectopic B-MYB (green). Nuclei were visualised with DAPI (blue). Transfected cells were counted and grouped according to whether they stained positive for activated caspase-3.

Table 3-1 Percentage of activated caspase-3 positive cells

Experiment	wt	G427	M624
1	27.0%	40.8%	35.2%
	30.9%	35.1%	40.4%
2	17.9%	22.2%	25.6%
	16.0%	23.1%	22.2%

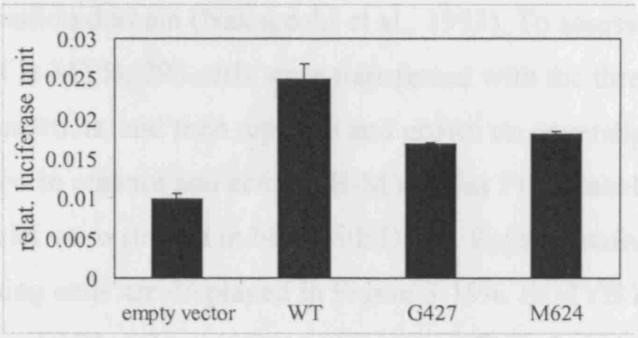
Comparison of activated caspase-3 positive cells between transfectants with wild type B-MYB or the G427 variant (Student's t-test, p= 0.047; n=4)
Comparison of activated caspase-3 positive cells between transfectants with wild type B-MYB or the M624 variant (Student's t-test, p= 0.001; n=4)
Total number of activated caspase-3 positive cells was 49.3% and 42.0% in experiment 1 and 39.8% and 36.9% in experiment 2.

3.5.3 Altered transactivation activity of B-MYB variants

The diversity in the apoptotic response of the different B-MYB forms might be caused by non-uniform activation of target genes. As the two SNPs are localised to the regulatory domain of B-MYB, it was interesting to test whether the SNPs would influence B-MYB transactivation. The regulatory domain has been shown to influence B-MYB transactivation capacity (Ziebold et al., 1997). Thus, the variants were transfected into SH-SY5Y neuroblastoma cells along with a construct containing the mim-1 promoter fused to the luciferase gene along with a CMV renilla plasmid. Mim-1 is a prototypic MYB target gene harbouring several MYB consensus binding sites (Ness et al., 1989). Transfected cells were cultured for 24 hours before dual luciferase reporter assays were performed. The experiment was performed in triplicate and the luciferase expression intensities were normalised with the renilla readings. Indeed, the two B-MYB variants display reduced transactivation activity compared to the wild type B-MYB form on the mim-1 promoter (Figure 3-18A).

To mimic a heterozygous situation, the B-MYB variants in combination with the B-MYB wild type were transfected in a 1:1 ratio into SH-SY5Y cells together with the luciferase and renilla plasmids. Even in combination with wild type B-MYB, the presence of the two variants resulted in reduced transactivation of the mim-1 promoter (Figure 19B).

A



B

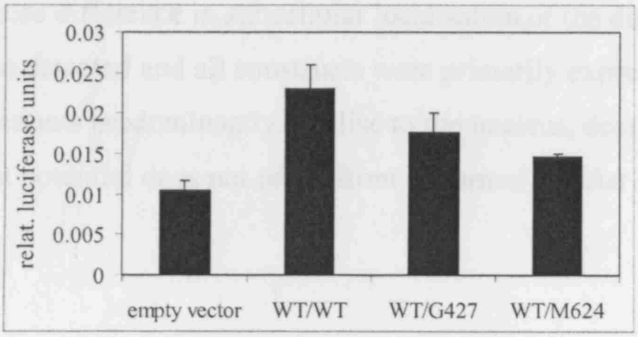
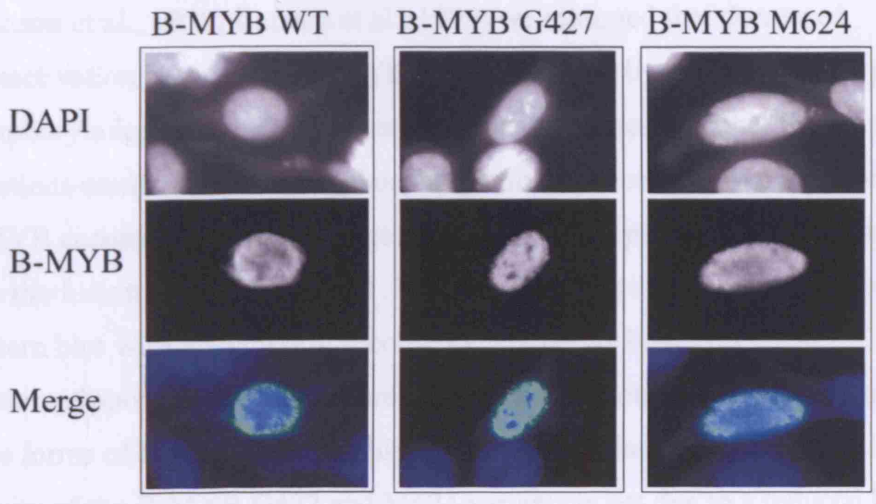


Figure 3-18 The transactivation capacity of the B-MYB variants is reduced
(A) Either of the B-MYB forms was transfected in triplicate into SH-SY5Y cells along with a mim-1 luciferase reporter construct and the CMV renilla expression vector. Cells were lysed and dual luciferase reporter assays were performed. Luciferase intensities were normalised to renilla expression intensities. Error bars indicated standard deviations. (B) Same as in A, except that equal amounts of wild type B-MYB in combination with either of the variants was used for transfections.

3.5.4 The B-MYB variants localise exclusively to the nucleus

B-MYB has been reported to localise exclusively to the nucleus (Robinson et al., 1996). However, as the two B-MYB variants cannot transactivate the mim-1 promoter as efficiently as the B-MYB wild type form it was important to ensure that the proteins were correctly expressed and localised to the nucleus. This was particularly important for the S427G substitution as it is close to the first B-MYB nuclear localisation domain (Nakagoshi et al., 1993). To assess subcellular localisation of B-MYB, 293 cells were transfected with the three B-MYB expression constructs, and then replated and grown on coverslips. Subsequently, cells were fixed in ethanol and ectopic B-MYB was FITC-labelled with an Xpress antibody. Nuclei were stained in blue with DAPI. Representative pictures of B-MYB expressing cells are displayed in Figure 3-19A. B-MYB localisation was visually assessed with a fluorescence microscope. Transfected cells were counted and categorised according to where B-MYB expression occurred in the cell (Figure 3-19B). No gross difference in subcellular localisation of the different B-MYB forms could be detected and all constructs were primarily expressed in the nucleus. As B-MYB variants predominantly localise to the nucleus, decreased transactivation potential does not result from abnormal cellular localisation.

A



B

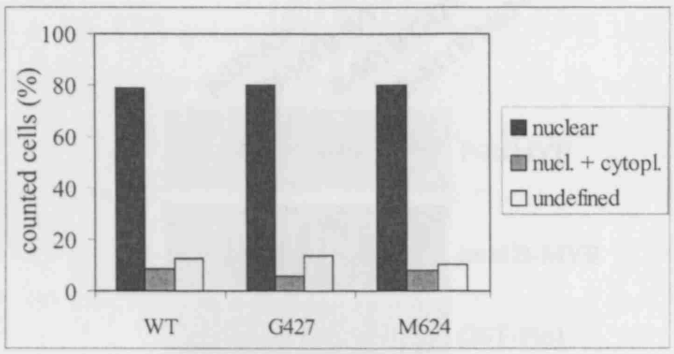


Figure 3-19 B-MYB variants are predominantly localised to the nucleus
(A) 293 cells were transfected with the different B-MYB forms and grown on coverslips. Cells were fixed and exogenous B-MYB was detected with an antibody specifically binding to tagged B-MYB (shown in green). Nuclei were stained with DAPI (blue). Representative immunofluorescence stainings are shown for the different B-MYB forms (B) Quantification of the experiment in A. Transfected cells were counted and localisation of exogenous B-MYB variants was assessed by microscopic analysis.

3.5.5 The different B-MYB forms display a similar overall phosphorylation status

Because phosphorylation has been previously identified to be an important post-translational modification of B-MYB, vital for regulating its transcriptional ability (Johnson et al., 1999; Bartsch et al., 1999) we reasoned that decreased transactivation potential of B-MYB could be caused by reduced overall protein phosphorylation in the B-MYB variant proteins. To test whether the point mutations could affect overall phosphorylation, 293 cells were transfected with the B-MYB construct or an empty vector as control and protein lysates were incubated with the fusion protein GST-Pin1. Purified phosphoproteins were analysed by western blot with an antibody specific to exogenous B-MYB (Figure 3-20). The fraction of phospho-B-MYB as well as the level of total B-MYB was similar for all three forms of B-MYB. Hence, this assay suggests that reduced transactivation activity of the B-MYB G427 and M624 variants is not due to a reduced level of phosphorylation.

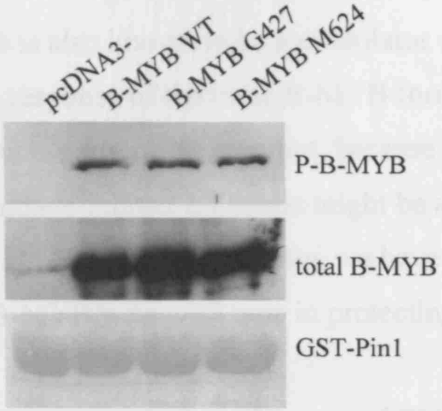


Figure 3-20 The B-MYB variants are similarly phosphorylated

293 cells were transfected with the different B-MYB forms. Protein lysates were subjected to a GST-Pin-1 pull down and purified proteins were subjected to western blot analysis. Phosphorylated B-MYB (P-B-MYB) and input B-MYB were detected with an antibody specifically detecting exogenous B-MYB.

3.5.6 Discussion

The most frequent cause of heritable cancer is based on polymorphic variations in the DNA sequence, particularly when genetic differences affect proteins regulating cell cycle control, apoptosis or angiogenesis (Imyanitov et al., 2004).
Even though point mutations are the most minimal polymorphic sequence alterations possible, several studies have identified significant functional differences among proteins with a single amino acid change (Aebersold et al., 2003; Sullivan et al., 2004; Bergamaschi et al., 2006). Single point mutations in B-MYB have previously been shown to considerably alter protein function. For example, introduction of a single point mutation into the *Drosophila* orthologue of B-MYB, whereby a glycine was substituted by a serine in the C-terminus, resulted in the failure to successfully complete cell division (Katzen et al., 1998). Therefore, it was interesting to assess whether the novel B-MYB variants were functionally different.

A well studied SNP in p53 is present at codon 72 and has been shown to modulate the cell's response to apoptosis (Bergamaschi et al., 2003; Dumont et al., 2003; Bergamaschi et al., 2006). While an arginine at position 72 induces high levels of apoptosis following the application of chemotherapeutic drugs, cell death in

response to chemotherapeutic drugs is less pronounced in cells harbouring a proline at position 72. As B-MYB is also known to be a modulator of cell survival, it was obvious to investigate the response of the three B-MYB forms to apoptosis-inducing agents. Apoptosis is particularly relevant, because it has been suggested that the reduced rate of damage-related apoptosis might be associated with elevated cancer risk (Imyanitov et al., 2004). Consequently, we have compared the ability of the two minor alleles of B-MYB with wild type in protecting cells from ROS-induced apoptosis.

Genomic DNA is constantly exposed to a wide array of DNA damaging agents, which can arise from endogenous sources as products of normal cellular metabolism caused by hydrolysis, lipid peroxidation and the formation of ROS (Cooke et al., 2003). Electron transport chains all leak electrons resulting in superoxide formation. Superoxides are precursors to different other ROS that have the potential to attack DNA and produce many forms of DNA damage (Cooke et al., 2003).

Cell cycle profiles of propidium iodide stained cells exposed to H_2O_2 showed that all three forms of B-MYB had a protective effect towards ROS-induced cell death compared to empty plasmid transfected cells. Permanent modification of DNA resulting from oxidative damage represents the first step involved in mutagenesis and carcinogenesis (Valko et al., 2007). Importantly, a reduced rate of DNA damage-induced apoptosis has been proven to be associated with elevated cancer risk (Imyanitov et al., 2004) and since B-MYB protects cells from ROS-induced apoptosis, B-MYB could be a factor contributing to the generation of cancer. Furthermore, analysis of cell cycle profiles of H_2O_2-treated cells revealed that the two B-MYB variants protected cells less efficiently than the wild type form of B-MYB, despite expression of the three B-MYB forms being equal. To further evaluate the effect of the three B-MYB forms on cell survival, cell death was assessed on the single cell level. Thus, we specifically measured ROS-induced apoptosis by assessing the cleavage of caspase-3. On the single cell level it became apparent that the wild type form of B-MYB confers greater protection from H_2O_2-induced cell death than the G427 and M624 variants. The number of activated caspase-3 positive cells may represent an underestimate, as many cells in late stages of apoptosis following H_2O_2-treatment detached from the microscope slide and could not be included in the counting.

To understand why the two B-MYB variants are less protective in response to apoptosis evoked by ROS, we assessed the transactivation potential of the different B-MYB forms. Dual luciferase reporter assays with the mim-1 promoter and the different B-MYB alleles, have ascertained that both the novel B-MYB variants displayed reduced transactivation activity compared to the wild type form. B-MYB G427 and M624 transactivated the mim-1 promoter only about half as efficiently as the wild type form when comparing transactivation above basal levels.
The B-MYB variants in combination with the B-MYB wild type, mimicking a heterozygous situation, were also able to remarkably decrease transactivation of the mim-1 promoter. Expression of a variant alone transactivated the mim-1 promoter to a similar extent as in combination with wild type. Hence, it appears that the variants are dominant over wild type B-MYB.

The mim-1 promoter is important in haematopoiesis (Ness et al., 1989) and we have used it as a prototypic MYB responsive promoter. However, in the future it will be interesting to assess the transactivation potential of the variants on a broad spectrum of B-MYB target genes particularly the ones known to be involved in the regulation of cell survival. It is likely that on other promoters the B-MYB variants may more efficiently activate or repress target genes. Despite both the minor alleles of B-MYB transactivate the mim-1 promoter similarly, it can nevertheless not be excluded that the two B-MYB variants transactivate some promoters differently. In an extreme case, it can be imagined that while B-MYB G427 might greatly transactivate a particular promoter, S624 might have a repressive effect on the same promoter. The best way to clarify differential gene expression induced by the diverse B-MYB alleles would be to perform gene chip analysis from cell lines each expressing only one of the B-MYB forms.
Reporter assays with the mim-1 promoter do not conclusively explain why the two B-MYB variants less effectively protect cells from apoptosis. Nevertheless, these experiments demonstrate as a proof of principle, that the B-MYB variants are able to modulate gene expression differently. This suggests that different degrees of target gene expression, possibly among other reasons, might contribute to different regulation of survival.

Why do the two B-MYB variants less efficiently regulate the mim-1 promoter? SNPs in other transcription factors have been demonstrated to affect subcellular localisation. For example, a greater proportion of the Arg72 variant of p53 localises to the mitochondrion upon apoptotic stimuli compared to the Pro72 polymorphism (Dumont et al., 2003). It is thus conceivable that the B-MYB polymorphisms could also affect its distribution in the cell. In support of this hypothesis Tashiro et al. have shown that B-MYB is localised to the cytoplasm when the NLS is deleted (Tashiro et al., 2004). Impairment of the nuclear transport of the B-MYB variants would explain less efficient transactivation of the mim-1 promoter. Even though the B-MYB polymorphisms do not occur in the NLS of B-MYB we sought to determine the subcellular localisation of all three B-MYB forms. Ectopically expressed B-MYB was stained and microscopic analysis was used to categorise B-MYB localisation. Apart from a few exceptions, all three polymorphic forms of B-MYB exclusively localised to the nucleus and no apparent difference between the localisation of the B-MYB variants could be detected. Thus, aberrant localisation of the B-MYB variants is not involved in decreased transactivation potential of the mim-1 promoter.

We also assessed overall B-MYB phosphorylation even though both amino acid substitutions do not affect amino acids known to be involved in B-MYB phosphorylation. Altered protein conformation could be caused by the amino acid substitutions and thus affect B-MYB phosphorylation. A change of conformation is particularly likely for the S417G SNP, as glycine is non-chiral and might therefore contribute to a change in protein conformation. However, all three B-MYB forms were similarly phosphorylated.

After all, two possibilities seem to be most likely to influence B-MYB variant transactivation as well as regulation of cell survival. Firstly, conformational changes might alter protein function. The best way to address this option would be by structural approaches such as NMR or X-ray crystallography. However, determining the tertiary structure of the B-MYB C-terminus seems to be problematic, as so far NMR spectroscopy has only allowed determination of the structure of two repeats of the DBD (McIntosh et al., 1998). The other possibility is that the amino acid changes in B-MYB could provoke alterations in B-MYB

binding to interaction partners. Alteration of polymorphism-induced binding partner specificity seems to be a concurrent theme as several examples exist in the literature describing this phenomenon. For example it has been observed that the Arg72 form of p53 preferentially associates with p73. It is broadly recognised that p53 mutants inhibit apoptosis as well as p73. Interestingly, p53 mutants of the Arg72 genotype were shown to inhibit p73-mediated apoptosis more effectively than the p53 Pro72 form (Bergamaschi et al., 2003). Conversely, wild type p53 induces apoptosis in response to a plethora of stimuli and importantly, the wild type p53 Pro72 variant is less active at inducing apoptosis than the Arg72 allele. Interestingly, the Pro72 form of p53 was found to be bound and its activity regulated more effectively by the p53 inhibitor iASPP compared to the p53 Arg72 polymorphism (Bergamaschi et al., 2006). Bottini et al. have also reported a SNP in the lymphoid protein tyrosine phosphatase LYP. The minor allele is more frequent in individuals with type 1 diabetes and is impaired in complex formation with the binding partner Csk, an important regulator of T-cell receptor signalling (Bottini et al., 2004).

Many other reasons could be envisaged affecting B-MYB transactivation. Various p53 tumour-associated point mutations result in the disruption of the DNA binding ability of p53 (Latonen and Laiho, 2005). A similar situation is unlikely in the case of the B-MYB variants, firstly because all three polymorphic forms of B-MYB have been shown to transactivate target genes with a MBS and must therefore bind DNA. Secondly, both SNPs occur in the B-MYB regulatory domain and should consequently not affect DNA binding, unless the amino acid alterations would influence protein conformation or disrupt interactions with other proteins supporting B-MYB DNA binding.

As the M624 variant of B-MYB results in a new ATG codon at position 624, it is intriguing to speculate that this nucleotide substitution could create an alternative translational start codon resulting in a truncated protein. This was observed for p53 whose sequence contains an alternative start codon resulting in an N-terminally truncated isoform of p53 named p53/47. Increased expression of the alternatively translated p53/47 stabilises p53 and alters the expression levels of p53 target genes (Yin et al., 2002). However, a smaller isoform of B-MYB could neither be detected in cell lines heterozygous for the M624 SNP as well as in cell lines overexpressing

the pcDNA3B-MYB M624 construct in western blot (data not shown). Even though an antibody was used that recognises the C-terminal part of B-MYB, it is still likely that this truncated isoform is only expressed under specific conditions or that it is expressed at low levels making it difficult to detect it by western blot. It might be important to address this possibility with more sensitive methods such as by radioactively labelling cellular proteins. A truncated form of B-MYB could act as an inhibitor of full-length B-MYB function and might thus readily explain the lowered transactivation potential as well as the reduced ability to rescue cells from oxidative stress-induced apoptosis of the M624 B-MYB variant.

3.6 Assessment of B-MYB variant frequency in neuroblastoma patients

3.6.1 Background

The cause of most diseases is complex and influenced by various environmental as well as genetic factors. All genetic variations are due to mutational processes, which occur at different rates in different parts of the genome and in the germ line. SNPs are one form of sequence variation in the human genome and they are by far the most abundant. SNPs located in non-coding regions of the gene can affect transcriptional regulation and ultimately modulate gene expression. Such polymorphisms are commonly referred to as regulatory SNPs (rSNPs). However, when a SNP occurs in a coding region, it is called a cSNP and such polymorphisms are most likely to affect gene function. The greatest proportion of cSNPs consists of synonymous SNPs, which contain a base pair substitution that despite altering the codon sequence still encode for the same amino acid. Most relevant for functional differences are so called non-synonymous SNPs. These SNPs harbour an amino acid substitution, which in some cases even results in protein truncation (Figure 3-21).

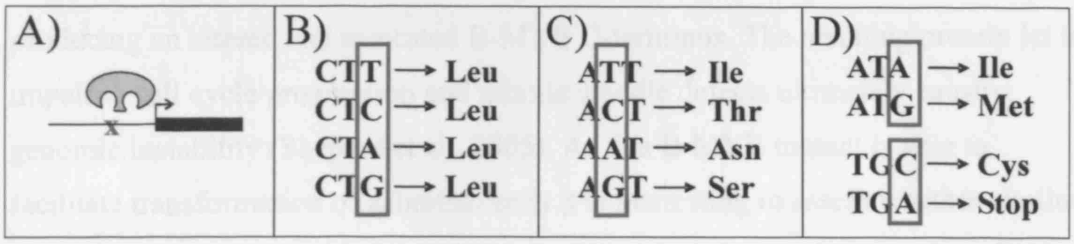


Figure 3-21 Schematic overview of different classes of SNPs

(A) SNPs in the untranslated region of a gene can influence protein expression by creating or deleting binding sites for transcription factors. (B) Various triplets encode a single amino acid. Nucleotide changes resulting in silent mutations are called synonymous. (C) Some point mutations change codons in a way that affects the resulting amino acid. Such SNPs are called non-synonymous. (D) Some non-synonymous SNPs generate truncated proteins by creating alternative translation start sites or premature stop codons.

Many research groups have focused their attention on genetic association analyses and the functional consequences of SNPs. Importantly, polymorphisms in the coding region of various genes were shown to cause or contribute to different diseases (Bottini et al., 2004; Saleh et al., 2004) including cancer (Levy-Lahad et al., 2001; Sullivan et al., 2004; Kono and Chen, 2005; Stacey et al., 2006). Mapping the SNP genotype might provide a foundation for assessing the susceptibility of an individual to disease and might support the choice of therapies.
While sequencing the coding region of the B-MYB gene we detected two non-synonymous SNPs in the B-MYB coding region. In the previous chapter we assessed functional differences among the different B-MYB forms, because only what we refer to as the wild type form of B-MYB has been functionally assessed to date. So far, only little information is available on the frequency and the distribution of these SNPs in the population. Thus, in this chapter I aimed to determine the frequency of the different B-MYB forms in healthy subjects and in neuroblastoma patients.

3.6.2 Setup of a large scale screening method for B-MYB and preliminary analysis of neuroblastoma samples

Shepard et al. detected a B-MYB loss of function mutant in a genetic screen in zebrafish. The alteration consisted of a point mutation in an intron boundary region producing an altered and truncated B-MYB C-terminus. The resulting protein let to impaired cell cycle progression and mitotic spindle defects ultimately causing genomic instability (Shepard et al., 2005). As this B-MYB mutant is able to facilitate transformation of zebrafish cells it is interesting to assess whether similar B-MYB mutations occur in human cancer. Since there is no other report available where potential B-MYB sequence alterations have been investigated, we sought to screen B-MYB for sequence variations in different neuroblastoma cell lines and in a panel of primary neuroblastoma tumour samples.
We have previously screened B-MYB by direct sequencing but as we attempted to analyse a larger number of samples, we needed to adapt a more cost-effective and rapid method. Accordingly, we have adjusted a method based on heteroduplex analysis to screen for B-MYB sequence alterations. Heteroduplex analysis allows

identifying mismatches in double-stranded DNA by detecting altered electrophoretic migration behaviour under non-denaturing gel conditions. Mismatch containing DNA fragments have slower gel mobility than homoduplexes due to bulges created by the mismatching base pairs.
Samples were electrophoretically separated and analysed on the MegaBACE 1000 instrument, a multi-capillary based DNA fragment analyser. The sensitivity of this multiplex capillary heteroduplex analysis (MCHA) method has been described to allow reliably screening a large number of samples of low DNA content (Hoskins et al., 2003). While conventional heteroduplex analysis is performed by gel electrophoresis, for MCHA, the DNA fragments of interest are fluorescent-labelled by PCR, de- and renatured, electrophoretically separated on a capillary-injected gel matrix and eventually their migration pattern is detected with a laser system.
De- and renaturing of fluorescently labelled PCR products allows the formation of heteroduplexes. While not needed for heterozygous samples, homozygous samples must be mixed with reference DNA to form mutant homoduplexes, wild type homoduplexes and two heteroduplex species (Figure 3-22).

Mismatches were previously detected in fragments of about 500 base pairs with reliable sensitivity (Hoskins et al., 2003) whereby mismatches within 50 base pairs of the end of the PCR product have been described to be difficult to detect (Rozycka et al., 2000). Hence, we designed primer pairs along the coding sequence of B-MYB flanking approximately 500 base pairs with neighbouring primers overlapping about 100 base pairs (Figure 3-23A). One of the primers of a pair was FAM-labelled thus in turn fluorescently-labelling the resulting PCR product.

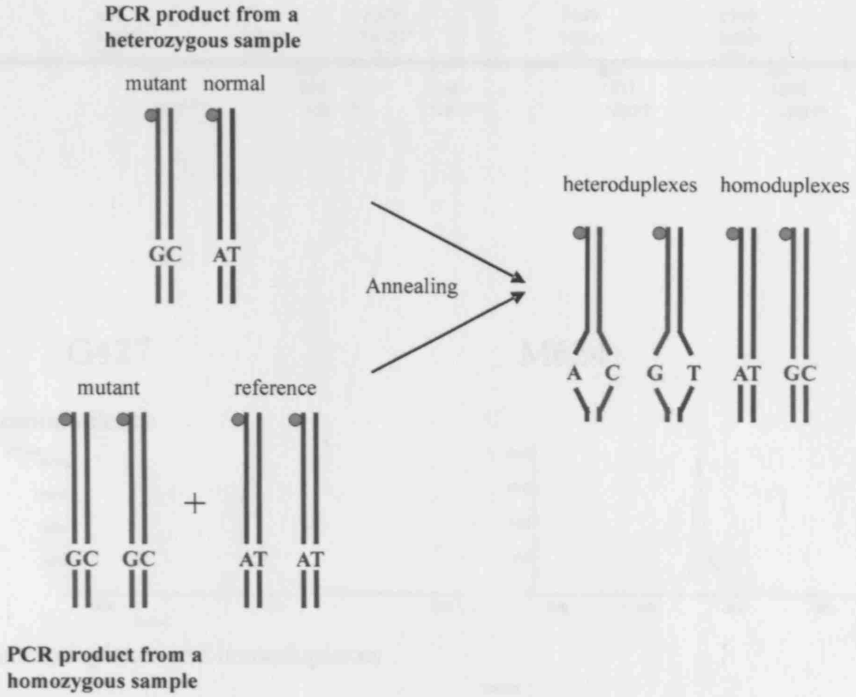
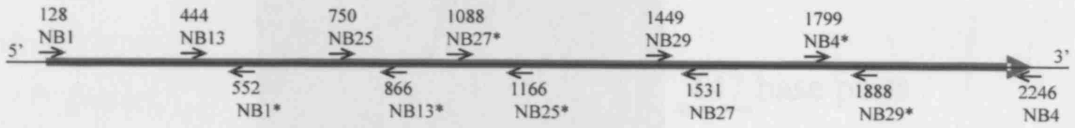


Figure 3-22 Schematic overview of the principles of heteroduplex analysis
Patient DNA was PCR amplified and Fam-labelled. PCR products were de- and renatured before subjected to heteroduplex analysis. Heterozygous samples form hetero- and homoduplexes, while homozygous mutant samples only form homoduplexes. Therefore, each sample is analysed on its own and in combination with reference DNA, which allows detection of homozygous mutant samples. Green circles represent the fluorochrome Fam.

A



B

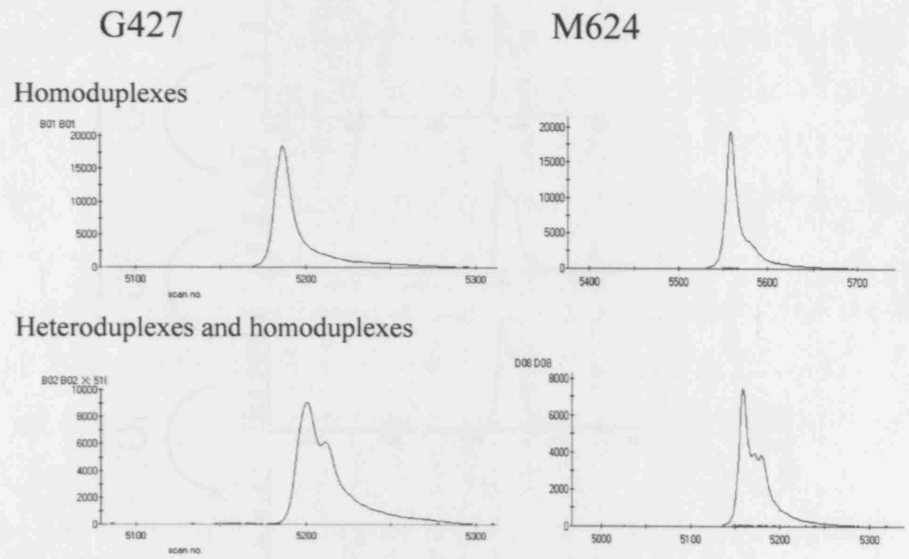


Figure 3-23 Strategy for analysing cDNA samples for B-MYB sequence variations by MCHA

(A) For cDNA samples, primers were designed along the coding region of B-MYB. Each primer pair amplified a sequence of about 500 bases and adjacent primer pairs overlapped about 100 bases. One primer of a pair was Fam-labelled, which is represented with an asterisk. The number above a primer indicates the position of the first nucleotide. (B) Representative chromatographs of fluorophor-labelled PCR products from cDNA each being heterozygous for one of the SNPs. The primer pair NB27 amplifies the region between nucleotide 1088 and 1531, which contains the G427 SNP and the primer pair NB4 spans the region between nucleotide 1799 and 2246 including SNP M624. Relative fluorescence intensities are shown in the y-axis. The x-axis is the time after initial sample injection and increases from left to right.

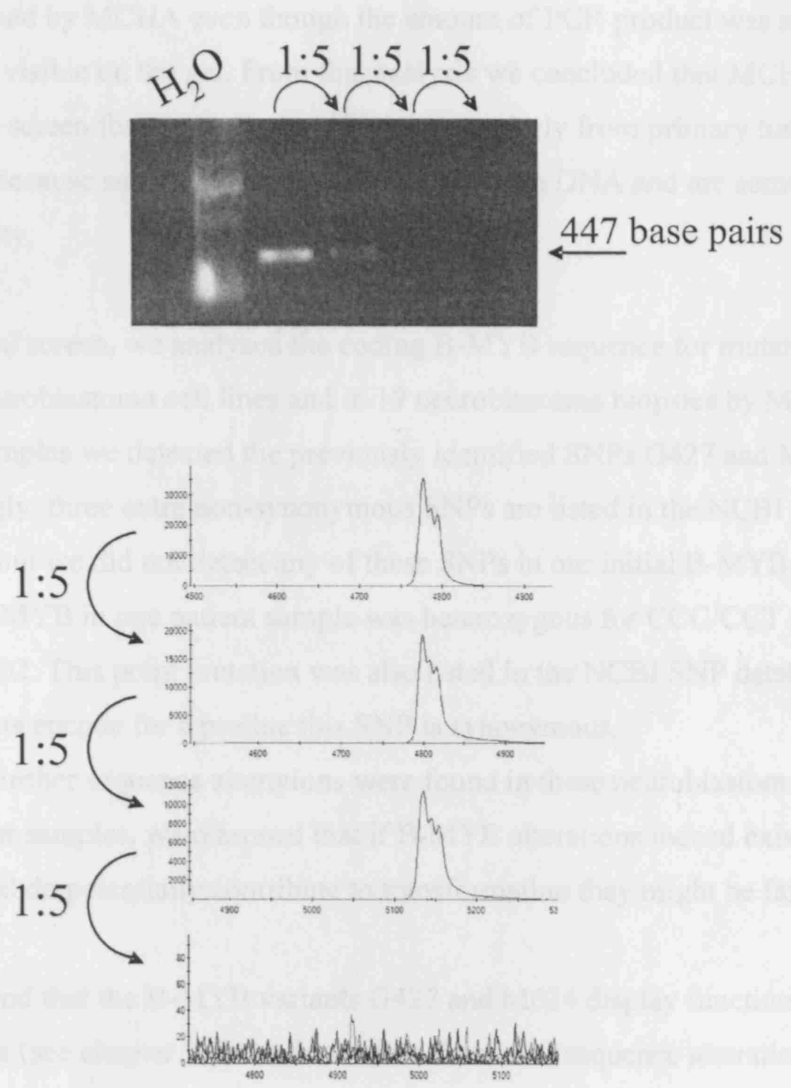


Figure 3-24 Assessing the sensitivity of MCHA

(A) A fragment of B-MYB containing the SNP M624 was amplified from the cDNA of SH-SY5Y cells with the primer pair NB4 by PCR. Note that this cell line is heterozygous for the SNP. A dilution series of the PCR product was assessed on an agarose gel. (B) The same dilution series was evaluated by MCHA. Relative intensity of fluorescence is indicated in the y-axis.

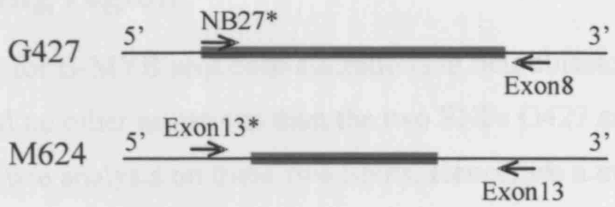
Sensitivity of the method was tested by PCR-amplifying cDNA from SH-SY5Y cells with the primer pair NB4. The PCR product was diluted as indicated and visualised by electrophoretic separation on an agarose gel (Figure 3-24A). The same amounts that were assessed on the agarose gel were subjected to MCHA and the signals obtained are displayed in Figure 3-24B. Interestingly, clear signals were

still obtained by MCHA even though the amount of PCR product was so little that it was not visible on the gel. From this analysis we concluded that MCHA is a good method to screen for sequence alterations, particularly from primary tumour biopsies, because such samples often consist of little DNA and are sometimes of poor quality.

In an initial screen, we analysed the coding B-MYB sequence for mutations in 13 human neuroblastoma cell lines and in 19 neuroblastoma biopsies by MCHA. In several samples we detected the previously identified SNPs G427 and M624. Interestingly, three extra non-synonymous SNPs are listed in the NCBI SNP database, but we did not detect any of these SNPs in our initial B-MYB sequence screen. B-MYB in one patient sample was heterozygous for CCC/CCT at codon position 302. This point mutation was also listed in the NCBI SNP database and as both triplets encode for a proline this SNP is synonymous.
Since no further sequence alterations were found in these neuroblastoma cell lines and tumour samples, we reasoned that if B-MYB alterations indeed exist in this tumour and do potentially contribute to transformation they might be fairly rare.

As we found that the B-MYB variants G427 and M624 display functional differences (see chapter 3.5) we focused on these two sequence alterations and attempted a case-control study to determine the frequency of the B-MYB variants in neuroblastoma samples compared to samples of healthy control subjects. Both our neuroblastoma and control samples were provided as either cDNA or genomic DNA samples and therefore we designed two new primer pairs allowing to analyse B-MYB from genomic DNA samples and thus detecting SNP G427 in exon 8 and SNP M624 in exon 13 (Figure 3-25A). The shape of the peaks obtained from the chromatographs of the homo- and heteroduplexes depends on the position of the mismatch and the length of the PCR product and therefore different peaks were observed from genomic DNA than from cDNA samples for the same SNPs (Figure. 3-25B).

A



B

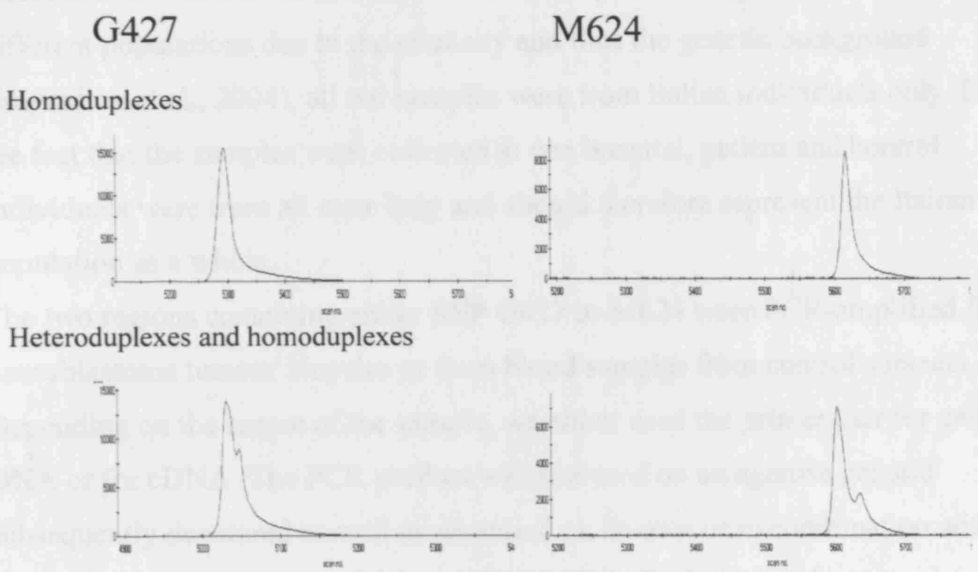


Figure 3-25 Strategy for analysing B-MYB sequence variations from genomic DNA samples by MCHA

(A) For genomic DNA samples, only the two regions containing the identified SNPs were assessed. SNP G427 is in exon 8 of B-MYB and was amplified with the primers NB27 and Exon8. SNP M624 occurs in exon 13 of B-MYB and the primer pair Exon13 was used to identify it. The asterisk symbolises the fluorophor Fam. (B) Representative chromatographs of the two SNPs amplified from genomic DNA are shown. The x-axis represents the time after sample injection and the y-axis represents the relative fluorescence intensity.

3.6.3 Assessing the frequency of two SNPs in the B-MYB coding region

As searching for B-MYB sequence alterations in neuroblastoma patients and cell lines revealed no other mutations than the two SNPs G427 and M624, we focused further sequence analysis on these two SNPs. Hence, we aimed to understand whether the presence or absence of these novel B-MYB variants could indicate a predisposition for neuroblastoma. In order to do so, we have sourced 130 DNA samples from neuroblastoma patients and 98 samples from healthy subjects. Because it is well known that the SNP frequency can change dramatically among different populations due to the ethnicity and thus the genetic background (Imyanitov et al., 2004), all our samples were from Italian individuals only. Despite the fact that the samples were collected at one hospital, patient and control individuals were from all over Italy and should therefore represent the Italian population as a whole.

The two regions containing either SNP G427 or M624 were PCR-amplified from neuroblastoma tumour biopsies or from blood samples from control subjects. Depending on the nature of the sample, we either used the primer pair for genomic DNA or for cDNA. The PCR product was analysed on an agarose gel and subsequently denatured as well as renatured on its own or in combination with reference DNA before it was analysed by MCHA. Emission peaks were examined and samples resulting in abnormal emission peaks were reamplified with unlabelled primers and confirmed by sequencing.

When corresponding blood samples were available for tumour biopsies, we tested whether the sequence alterations were indeed SNPs and not somatic mutations. Notably, results from patient blood samples concurred in all cases with the data from tumour biopsies.

We have pooled the results from our control samples with those obtained from another 132 control samples screened in the laboratory of Prof. Bruno Calabretta, University of Modena, Italy. Our combined results revealed that the two minor alleles G427 and M624 of B-MYB are common in normal human subjects. Notably, our screen showed that these B-MYB variants are much less frequent in neuroblastoma patients (Table 3-4). 20.8% of the neuroblastoma patients carried the

G427 allele, in contrast, 27.8% of the control samples were either heterozygous or homozygous for the G427 SNP, but this difference is not statistically significant (Table 3-2). On the other hand 5.4% of the neuroblastoma patients were positive for the M624 SNP, whereas 13.9% of the control group have this allele. When analysed by chi-square test, the M624 allele was significantly more frequent in the control population than in the neuroblastoma patient population compared to wild type B-MYB with a p-value of 0.02 (OR, 0.35; 95% 0.15 to 0.82) (Table 3-3).

Table 3-2 Association between the G427 B-MYB polymorphisms and incidence of neuroblastoma

Genotype	neuroblastoma		control		
WT/WT	103	(79.2%)	166	(72.2%)	
G427/WT	27	(20.8%)	63	(27.4%)	p = 0.2
G427/G427	0	(0%)	1	(0.4%)	p = 0.81

Table 3-3 Association between the M624 B-MYB polymorphisms and incidence of neuroblastoma

Genotype	neuroblastoma		control		
WT/WT	123	(94.6%)	198	(86.1%)	
M624/WT	7	(5.4%)	32	(13.9%)	p = 0.02
M624/M624	0	(0%)	0	(0%)	

p=0.02 chi square test, Odds ratios 0.35, 95% Confidence Interval 0.15 to 0.82

Table 3-4 Association between any B-MYB polymorphism and incidence of neuroblastoma

Genotype	neuroblastoma		control		
WT/WT	98	(75.4%)	141	(61.3%)	
any variant	32	(24.6%)	89	(38.6%)	p = 0.01

p=0.01 chi square test, Odds ratios 0.52, 95% Confidence Interval 0.32 to 0.84

Overall, there is a significant difference between the two populations when comparing the presence of the wild type form of B-MYB to the occurrence of any of the two B-MYB variants with a p-value of 0.01 (OR, 0.52; 95% 0.32 to 0.84) (Table 3-4). This result suggests that individuals with one or both the B-MYB variants are less likely to suffer from neuroblastoma.

3.6.4 Results from our collaborators

As we were interested whether the distribution of the minor B-MYB alleles differed only in neuroblastoma patients with respect to the control population, Prof. Calabretta's group additionally analysed a panel of CML patient samples. Consistent with our findings, the B-MYB variants are less frequent in CML patients (Table 3-5). Out of 126 CML patients, 16.7% were positive for the G427 SNP and in 12.7% of the CML patients the presence of the M624 SNP was established. The G427 allele was significantly more frequent in the control population than in the CML patient population compared to wild type B-MYB with a p-value of 0.03 (OR, 0.53; 95% 0.3 to 0.91) (Table 3-5), but the difference in the frequency of the M624 SNP compared to the frequency of the control population was not significant (Table 3-6). This is the opposite of what we observed with neuroblastoma patients. Nevertheless, similar to our findings with neuroblastoma patient samples, on the whole there is a significant difference between the CML and control populations in the incidence of the wild type form of B-MYB to the frequency of any B-MYB variant with a p-value of 0.02 (OR, 0.54; 95% 0.33 to 0.87) (Table 3-7). Therefore, this result implies that when a subject harbours one of two or both of the B-MYB variants they are less likely to acquire CML.

Table 3-5 Association between the G427 B-MYB polymorphisms and incidence of CML

Genotype	CML		control		
WT/WT	105	(83.3%)	166	(72.2%)	
G427/WT	21	(16.7%)	63	(27.4%)	p = 0.03
G427/G427	0	(0%)	1	(0.4%)	p = 0.81

p=0.03 chi square test, Odds ratios 0.53, 95% Confidence Interval 0.3 to 0.91

Table 3-6 Association between the M624 B-MYB polymorphisms and incidence of CML

Genotype	CML		control		
WT/WT	110	(87.3%)	198	(86.1%)	
M624/WT	16	(12.7%)	32	(13.9%)	p = 0.86
M624/M624	0	(0%)	0	(0%)	

Table 3-7 Association between any B-MYB polymorphism and incidence of CML

Genotype	CML		control		
WT/WT	94	(74.6%)	141	(61.3%)	
any variant	32	(25.4%)	89	(38.7%)	p = 0.02

p=0.02 chi square test, Odds ratios 0.54, 95% Confidence Interval 0.33 to 0.87

3.6.5 Discussion

Growing interest in SNPs has led to diverse studies linking polymorphic alleles to predispositions to a variety of diseases including cancer. For example, a SNP in caspase-12 results in a read-through instead of a stop codon. This longer form of caspase-12 is restricted to the African populations where it is associated with increased susceptibility to sepsis (Saleh et al., 2004). While this is an extreme

example of a polymorphism, other researchers have described cases where SNPs that do neither result in a truncated nor an elongated protein are nevertheless significantly associated with increased risk for a particular disease. Such an example is a polymorphism in the receptor tyrosine kinase Met, which results in constitutive receptor activation and this polymorphism is associated with increased incidence of throat cancer (Aebersold el al, 2003).

Whilst investigating the role of B-MYB in human cancer, we observed that two non-synonymous polymorphic variants (S427G and I624M) in the B-MYB exonic region are frequent in human cancer cell lines.
A search in the NCBI SNP database reveals that only a few SNPs occur in the coding region of B-MYB but conversely a plethora of SNPs have arisen in the non-coding region of B-MYB. One SNP was recorded in the 5'- and four in the 3'-untranslated region of B-MYB. Additionally, a vast number of SNPs are located in the intron sequences of B-MYB. The huge amount of SNPs in untranslated regions or intron sequences of the B-MYB gene might complicate their analysis, nevertheless it might be interesting to draw the attention to these polymorphisms as they could contribute to the regulation of B-MYB. Such a regulatory SNP was identified in the upstream regulatory elements of the α-globin gene cluster. It was proposed that the minor allele generates a new binding site for the transcription factor GATA-1. Presence of the minor allele coincided with the downregulation of α-globin genes and was associated with α-thalassemia (De Gobbi et al., 2006). Similarly, a SNP in the 5'-untranslated region of the RAD51 gene increased breast cancer risk in BRCA2 mutant carriers (Levy-Lahad et al., 2001). Moreover, a polymorphism located in the first intron of the MDM2 gene creates an improved binding site for the ubiquitously expressed transcription factor Sp1. This SNP results in increased MDM2 expression levels causing an attenuated p53 response. Consequently, this polymorphism was associated with generally accelerated tumour development (Bond et al., 2004).

Furthermore, five synonymous SNPs are distributed over exons 5, 6, 7 and 9 in the B-MYB gene. The analysis of synonymous SNPs is usually neglected as such polymorphisms are commonly believed to be functionally neutral. Regardless, accumulating evidence exists that synonymous SNPs may still alter protein function

as single base changes in specific recognition sequences have been previously implicated in affecting translational processes such as mRNA splicing (Cartegni et al., 2002). These findings indicate that it might have been worthwhile to include the analysis of synonymous SNPs in our screening for B-MYB polymorphisms. However, before assessing a vast array of samples for synonymous SNPs in the B-MYB coding region it might be wise to initially address whether translational abnormalities would indeed occur.

As further indicated in the NCBI SNP database, the B-MYB coding region harbours five non-synonymous SNPs in total. Interestingly, all non-synonymous SNPs are located in the B-MYB carboxy-terminal domain, which regulates the transactivation function of B-MYB. Both, the N341S and the P450R SNPs occur in exon 8 and the V595M SNP is located in exon 12 of B-MYB. Frequencies of these non-synonymous SNPs given in the NCBI SNP database indicate that they constitute very rare alleles in the human population. In support of the proposed rarity of these alternative variants, we did not detect their presence when we analysed the whole coding sequence of B-MYB in an initial screen in a panel of neuroblastoma cell lines as well as patient samples.
While no frequency data is available for the I624M polymorphism, a search in the NCBI SNP database confirmed that S427G is frequent in the human population. The suggested frequency of individuals heterozygous for this polymorphism ranges from 16.7% to 23.3% in American Caucasians and seems to be comparable in Asian and Hispanic populations. However, the presence of the homozygous genotype of the minor alleles is very rare in these populations. Interestingly, according to the SNP database the S427G SNP appears to be particularly prevalent in individuals of African descent, as almost 50% of these subjects are heterozygous carriers and about 8% are homozygote for the minor allele. Thus, the presence of the S427G polymorphism apparently varies with ethnicity and is maintained at different allelic frequencies across the population of the world. This is not unusual, as the ethnical background is an important determinant for genetic variability and the prevalence of certain SNPs has been reported to vary among particular populations (Saleh et al., 2004; Kono and Chen, 2005; Gu et al., 2006).

The B-MYB SNP frequencies from the NCBI database were generally determined with low sample numbers, in most groups not exceeding 30 samples. Thus, obviously greater sample numbers are needed to reliably determine the SNP frequency. Our Italian control population consisted of samples of 230 subjects and we determined a slightly higher frequency with 27.4% being heterozygous for S427G and 0.4% homozygous for the G427 polymorphism. On the other hand, we have determined that the I624M SNP is an unbalanced polymorphism with 13.9% Italian individuals being heterozygous carriers, whereas no homozygous individual was identified.

Prof. Calabretta's group contributed to the control sample screening and determined the frequency of the two B-MYB SNPs by HPLC. The frequencies of the G427 SNPs in the two independent control sub-populations were similar, with either 27.6% or 27.3%. This validates our approach to measure the frequencies by MCHA and the choice of the control samples. However, the M624 polymorphism was 8.2% in one control population and 18.2% in the other. This discrepancy might be ascribed to the relatively low frequency of this polymorphism and it indicates that a greater number of samples would be required to more reliably determine the frequency of this polymorphism.

In summary, our data demonstrate that both, the G427 and to a lesser extent the M624 allele are common in the Italian population. An advantage of frequently occurring SNPs is that statistical significance can be more readily achieved with relatively low numbers of cases and controls. This is particularly relevant for studies in rare cancers such as neuroblastoma where only a limited number of samples is available for experimental research. Despite the finding that polymorphisms in oncogenes, such as HRAS1 have been shown to significantly modulate the risk of human cancer, they have little impact as they are rarely found in the human population (de Jong et al., 2002). In contrast, the B-MYB polymorphisms investigated here are much more common and thus these variations may account for a higher population attributable risk.

We found that the two polymorphic B-MYB variants G427 and M624 less effectively confer protection from apoptosis and have the potential to regulate

MBS-promoters differently (compare chapter 3.5). Reduced apoptosis has been linked to increased cancer risk (Imyanitov et al., 2004) and therefore subjects carrying alleles with diminished anti-apoptotic function should have a reduced risk of developing neoplastic disease. Thus, we sought to determine whether such a correlation could be observed by comparing the frequency of the B-MYB polymorphisms in samples from a cohort of non-cancer Italian subjects with samples from neuroblastoma patients. The frequency of the alternative B-MYB alleles was determined from 130 neuroblastoma patient biopsies collected in Italian hospitals. While analysing cancer patient samples for their SNP status, we observed that the frequency of the G427 and M624 alleles was greatly reduced in subjects diagnosed with neuroblastoma.

In samples from neuroblastoma patients, the G427 allele occurs in 20.8% and the I624 allele in 5.4% of all cases. We found that when compared with the S427S homozygote, the heterozygous S427G genotype was not significantly associated with risk of neuroblastoma incidence and the homozygous G427G genotype did not occur at all in neuroblastoma patients. On the contrary, the presence of the I624M heterozygote genotype was associated with significantly ($p = 0.01$) decreased risk of neuroblastoma incidence (OR, 0.32; 95% 0.13 to 0.74). The combined effect of both the minor alleles occurring either as a heterozygote or as a homozygote genotype revealed a significant ($p = 0.01$) association with increased risk of neuroblastoma (OR, 0.52; 95% 0.32 to 0.84).

Conversely, in CML patients, statistical analysis indicated that subjects carrying the S427G heterozygote genotype had a significantly decreased risk ($p = 0.025$) of CML incident (OR, 0.49; 95% 0.28 to 0.86) and those carrying the I624M heterozygote had a lowered risk compared with the homozygous I624I genotype. Thus, while only the occurrence of the S427G heterozygote was significant, the presence of either one of the two minor B-MYB alleles is significantly ($p = 0.025$) associated with decreased risk of CML (OR, 0.54; 95% 0.33 to 0.87).

Other studies have investigated the combined effect of polymorphisms as well. For example, two non-synonymous SNPs within the MMP-9 gene were associated with the risk of developing lung cancer. While only one of the SNPs was significantly associated with predictive power for lung cancer, combination of the two polymorphisms revealed a greatly enhanced risk of lung cancer incidence in

individuals carrying either one or both of the polymorphisms in the MMP-9 gene (Hu et al., 2005).

Overall, our data provides evidence that the occurrence of the B-MYB major alleles is associated with an increased risk of developing neuroblastoma and CML, respectively. These findings indicate that the B-MYB polymorphisms G427 and I624 could constitute a marker for the probability of acquiring cancers such as neuroblastoma and CML.
Since the B-MYB polymorphisms were shown to have significant predictive power in two different types of cancers, it is conceivable that this observation is extendable on an even broader range of cancers. A more comprehensive meaning for the B-MYB SNPs in cancer would not be unique, as one single polymorphism in the MTHFR gene has also been linked to several different types of cancer, including colorectal (Kono and Chen, 2005), breast (Chen et al., 2005) and gastric cancer (Graziano et al., 2006).

In a majority of studies, the minor allele confers a certain risk for a particular predisposition (Levy-Lahad et al., 2001; de Jong et al., 2002; Hu et al., 2005; Stacey et al., 2006). However, a change from a serine to the less frequently represented glycine in the Rho guanine nucleotide exchange factor family member PDZ-Rho was associated with reduced risk of lung cancer among Mexican Americans (Gu et al., 2006). Likewise, SNPs in untranslated regions of the G protein signalling regulators RGS2 and RGS6 were linked to reduced bladder cancer risk (Berman et al., 2004). We report here a similar case where the presence of a minor allele of B-MYB appears to confer protection from neuroblastoma as well as CML incidence.

Frequent practice is to compare the occurrence of polymorphisms with other prognostic factors (Aebersold et al., 2003; Hu et al., 2005; Gu et al., 2006). We only possess accompanying prognostic patient data such as age, stage as well as amplification- or deletion status for a subset of neuroblastoma patients. This hampers the investigation of a potential correlation between neuroblastoma stages and B-MYB SNP status. However, even if we were in possession of full patient information for the whole cohort of our neuroblastoma patient samples we

nevertheless might not be able to conclusively correlate the B-MYB SNP status with other prognostic factors as the sample number might be to low.

Similar to other case-control studies, sample selection biases may lead to erroneous findings. The frequency of the B-MYB alleles was determined in two different laboratories with different methods using control populations from different origins. Both case and control samples were collected in hospitals that treat patients from all over Italy and neither the control nor the case individuals should be related. Therefore, our samples should be free of ethnical as well as inherited biases. Over and above, our control population should match the cohort of paediatric cases, even though our controls derive from adult donors. It has been proposed that it can be even advantageous to use control cases of individuals markedly older than the patient population as therefore it can be excluded that the control group would still be likely to succumb to the disease (Imyanitov et al., 2004).

Cancers depend on various factors and among them are genetic variations and specific interactions caused by these variations. The analysis of polymorphisms is important as elucidating genetic determinants of disease progression or treatment outcome might facilitate the identification of patients most likely to benefit from aggressive treatments. The results presented here suggest that determining the SNP status of B-MYB might be one potentially useful marker among others to determine the risk of acquiring cancers such as neuroblastoma, CML and possibly others. However, it will be important to verify the frequencies and distributions of the B-MYB polymorphisms in larger case and control populations, in other ethnical backgrounds and in further types of cancers.

4 CONCLUSION

Neuroblastoma is a paediatric cancer, which affects 1 in 100,000 children under the age of 15 years (Sawada et al., 1987; Bernstein et al., 1992). Treatment of advanced stage neuroblastoma patients involves the application of high doses of chemotherapeutic agents. This is associated with considerable overall toxicity. Despite these children have to endure aggressive treatment regimens, the overall mortality rate of advanced stage patients is very high (van Noesel 1997). Therefore, alternative approaches might improve or complement common treatment strategies. In order to test the possibility that downregulation of B-MYB could lead to reduced proliferation or even induce apoptosis of neuroblastoma cells, I have evaluated the efficacy of several RNAi target sequences, whereby I successfully identified a short sequence complementary to the human B-MYB mRNA that allowed significant downregulation of B-MYB in various cell lines. Surprisingly, reduction of the B-MYB expression levels could not be easily achieved in neuroblastoma cell lines, because the B-MYB protein is highly stable in these cells compared to other tumourigenic or normal cell lines. While these findings suggest that efforts aimed at specifically targeting B-MYB in neuroblastoma tumours might not be worth pursuing, it cannot be excluded that it might be easier to achieve downregulation of B-MYB in primary neuroblastoma tumour cells and that enhanced stabilisation of B-MYB is a feature acquired in culture. In contrast, preliminary data in this thesis has shown that downregulation of B-MYB might have therapeutic value in other cancers such as Ewing sarcoma. Downregulation of B-MYB with siRNA oligonucleotides was fairly achievable in these cells and, importantly, reduced levels of B-MYB prompted Ewing sarcoma cells to die.

The notion that B-MYB is important for proliferation and/or survival of neuroblastoma cells was firstly demonstrated by Raschella and colleagues using antisense techniques (Raschella et al., 1995). With our investigation we have extended these results and demonstrated that, it is not always possible to achieve B-MYB knockdown in neuroblastoma cell lines, because the protein is aberrantly stable.

Protein turnover is affected by the rate of proteolytic degradation and changes in protein stability can influence the transforming activity of oncogenic proteins. For example, it was hypothesised that prolonged stability of N-MYC might contribute to tumour progression in a manner similar to when its coding sequence is amplified (Cohn et al., 1990). Our data add support to the hypothesis that an important consequence of abnormal B-MYB stabilisation might be the increased survival of neuroblastoma cells in response to insults such as UV-induced DNA damage. Hence, non-degradable B-MYB could contribute to aggressive neuroblastoma behaviour by supporting the survival of tumour cells bearing genomic lesions that would otherwise be eliminated by apoptosis.

While assessing post-translational modifications of B-MYB, I noticed that endogenous B-MYB was less phosphorylated in neuroblastoma compared to Ewing sarcoma cells. As it is known that phosphorylation of B-MYB leads to its proteasomal destruction (Charrasse et al., 2000), hypophosphorylation of B-MYB might constitute a mechanism by which its half life is increased. This notion was confirmed by experiments showing that a phosphorylation deficient mutant of B-MYB is more stable than its wild type counterpart. Why B-MYB is hypophosphorylated in neuroblastoma cells is currently not clear. This could be caused by lack of a critical kinase or by increased activity of a phosphatase. These possibilities will be addressed in future experiments.

Whereas low levels of B-MYB phosphorylation provide an explanation for its increased stability, it was important to address the question whether hypophosphorylated B-MYB could still be involved in modulating cell survival. Overexpression of the phosphorylation deficient B-MYB mutant promoted cell survival to an extent similar to wild type B-MYB. Previous reports suggested that B-MYB exerts its anti-apoptotic function via direct activation of target genes (Grassilli et al., 1999; Cervellera et al., 2000; Lang et al., 2005). Thus, it is surprising that a phosphorylation deficient mutant promotes survival, taking into account evidence suggesting that phosphorylation is required to enhance B-MYB transactivation activity (Lane et al., 1997; Ziebold et al., 1997; Saville and Watson, 1998; Johnson et al., 1999; Muller-Tidow et al., 2001; Bessa et al., 2001). However, B-MYB phosphorylation may not be essential for the activation of all B-

MYB target genes, particularly of those activated by B-MYB in a MBS-independent fashion (Kamano and Klempnauer, 1997; Sala et al., 1999; Bartusel et al., 2005). Our finding that hypophosphorylated B-MYB is functionally active is somehow in agreement with previous work in which it was shown that the transforming ability of B-MYB is not enhanced but impaired in the presence of cyclin A (Masselink et al., 2001).

We speculated that increased stability of B-MYB could be achieved through mutations of its coding region. We ruled out gross mutational abnormalities in the coding sequence of B-MYB in several neuroblastoma cell lines and the only sequence abnormalities we detected were single point mutations in the regulatory domain of B-MYB. One amino acid change was found in the neuroblastoma cell line SK-N-AS, which harbours a serine instead of a glycine (G427) at codon 427. The other change involving a single amino acid was detected in SH-SY5Y cells where the isoleucine at position 624 is substituted with a methionine (M624). These mutations occur with high frequency in the human population and are therefore SNPs. We went on to investigate whether these amino acid changes could affect B-MYB stability. Although we demonstrated that the B-MYB polymorphisms were not causing changes in protein stability, nevertheless they were functionally distinct from the wild type protein. Consequently, our findings suggest that aberrant overexpression of the less functional B-MYB alleles might not be as detrimental as that of the more efficient major allele and that subjects carrying the minor alleles have a reduced risk of developing cancer. For this reason we investigated whether B-MYB SNPs could be detected in cancer patients, also considering that most of population-attributable cancer heritability is related to polymorphic variations (Imyanitov et al., 2004).

Genotyping the control population revealed that the SNP G427 occurs frequently in the Italian population whereas the M624 SNP is less common. While the allele M624 is significantly less frequent in neuroblastoma than normal subjects, the allele G427 is significantly less frequent in CML patients compared to normal subjects. Furthermore, the statistical significance increases when the whole group of cancer patients is compared to the healthy subjects and therefore tumour formation appears to be facilitated in the wild type B-MYB background. As the

presence of both the minor alleles was significantly lower in cancer patients than in controls, screening populations for the B-MYB polymorphisms might be useful to identify individuals that are at reduced or increased risk of developing neoplastic disease, including neuroblastoma.

In summary, the findings described in this thesis show that B-MYB overexpression can confer survival advantage and therefore aberrant stabilisation of B-MYB, as observed in several neuroblastoma cell lines, might result in B-MYB accumulation similar to when B-MYB is overexpressed. We conclude that the mechanism of B-MYB stabilisation described herein could contribute to tumourigenesis. Further research is necessary to understand whether aberrant B-MYB stabilisation is a consequence or an initiating event in transformation. Even though chemotherapy treatment significantly increases long term neuroblastoma survivors, a large number of children suffering from neuroblastoma still succumb to the disease (Westermann and Schwab, 2002). Hence, identifying factors that interfere with B-MYB stability might be useful to find new ways of suppressing neuroblastoma cell proliferation and enhance chemotherapeutic drug treatments.

While N-MYC amplification is one of the most powerful predictive features of neuroblastoma outcome, it only predicts aggressive behaviour of a subset of neuroblastomas (Brodeur et al., 1997). Hence, other prognostic factors indicative of neuroblastoma staging may ultimately ameliorate the risk assessment of neuroblastoma patients. Expression of B-MYB in primary neuroblastoma tumours was significantly associated with poor patients survival and it was proposed that B-MYB expression constitutes a prognostic factor that is able to complement the predictive value of N-MYC amplification (Raschella et al., 1999). It will be interesting to assess whether B-MYB polymorphisms have a predictive value on neuroblastoma outcome. This could allow to finely tuning therapeutic approaches to those patients at greater risk of developing aggressive and recurrent disease.

5 REFERENCES

Abate,C., Patel,L., Rauscher,F.J., III, and Curran,T. (1990). Redox regulation of fos and jun DNA-binding activity in vitro. Science *249*, 1157-1161.

Aebersold,D.M., Landt,O., Berthou,S., Gruber,G., Beer,K.T., Greiner,R.H., and Zimmer,Y. (2003). Prevalence and clinical impact of Met Y1253D-activating point mutation in radiotherapy-treated squamous cell cancer of the oropharynx. Oncogene *22*, 8519-8523.

Ahlbory,D., Appl,H., Lang,D., and Klempnauer,K.H. (2005). Disruption of B-myb in DT40 cells reveals novel function for B-Myb in the response to DNA-damage. Oncogene *24*, 7127-7134.

Almgren,M.A., Henriksson,K.C., Fujimoto,J., and Chang,C.L. (2004). Nucleoside diphosphate kinase A/nm23-H1 promotes metastasis of NB69-derived human neuroblastoma. Mol. Cancer Res. *2*, 387-394.

Amatschek,S., Koenig,U., Auer,H., Steinlein,P., Pacher,M., Gruenfelder,A., Dekan,G., Vogl,S., Kubista,E., Heider,K.H., Stratowa,C., Schreiber,M., and Sommergruber,W. (2004). Tissue-wide expression profiling using cDNA subtraction and microarrays to identify tumor-specific genes. Cancer Res. *64*, 844-856.

Ansieau,S., Kowenz-Leutz,E., Dechend,R., and Leutz,A. (1997). B-Myb, a repressed trans-activating protein. J. Mol. Med. *75*, 815-819.

Archambault,J. and Friesen,J.D. (1993). Genetics of eukaryotic RNA polymerases I, II, and III. Microbiol. Rev. *57*, 703-724.

Arsura,M., Introna,M., Passerini,F., Mantovani,A., and Golay,J. (1992). B-myb antisense oligonucleotides inhibit proliferation of human hematopoietic cell lines. Blood *79*, 2708-2716.

Attwooll,C., Lazzerini,D.E., and Helin,K. (2004). The E2F family: specific functions and overlapping interests. EMBO J. *23*, 4709-4716.

Axelson,H. (2004). The Notch signaling cascade in neuroblastoma: role of the basic helix-loop-helix proteins HASH-1 and HES-1. Cancer Lett. *204*, 171-178.

Ayer,D.E., Kretzner,L., and Eisenman,R.N. (1993). Mad: a heterodimeric partner for Max that antagonizes Myc transcriptional activity. Cell *72*, 211-222.

Bader,S.A., Fasching,C., Brodeur,G.M., and Stanbridge,E.J. (1991). Dissociation of suppression of tumorigenicity and differentiation in vitro effected by transfer of single human chromosomes into human neuroblastoma cells. Cell Growth Differ. *2*, 245-255.

Bagchi,A., Papazoglu,C., Wu,Y., Capurso,D., Brodt,M., Francis,D., Bredel,M., Vogel,H., and Mills,A.A. (2007). CHD5 is a tumor suppressor at human 1p36. Cell *128*, 459-475.

Bar-Shira,A., Pinthus,J.H., Rozovsky,U., Goldstein,M., Sellers,W.R., Yaron,Y., Eshhar,Z., and Orr-Urtreger,A. (2002). Multiple genes in human 20q13 chromosomal region are involved in an advanced prostate cancer xenograft. Cancer Res. *62*, 6803-6807.

Barberis,A. and Petrascheck,M. (2003). Transcription Activation in Eukaryotic Cells. Encyclopedia of Life Sciences.

Bartsch,O., Horstmann,S., Toprak,K., Klempnauer,K.H., and Ferrari,S. (1999). Identification of cyclin A/Cdk2 phosphorylation sites in B-Myb. Eur. J. Biochem. *260*, 384-391.

Bartusel,T. and Klempnauer,K.H. (2003). Transactivation mediated by B-Myb is dependent on TAF(II)250. Oncogene *22*, 2932-2941.

Bartusel,T., Schubert,S., and Klempnauer,K.H. (2005). Regulation of the cyclin D1 and cyclin A1 promoters by B-Myb is mediated by Sp1 binding sites. Gene *351*, 171-180.

Basilico,C., Ambrosetti,D., Fraidenraich,D., and Dailey,L. (1997). Regulatory mechanisms governing FGF-4 gene expression during mouse development. J. Cell Physiol *173*, 227-232.

Bauer,A., Savelyeva,L., Claas,A., Praml,C., Berthold,F., and Schwab,M. (2001). Smallest region of overlapping deletion in 1p36 in human neuroblastoma: a 1 Mbp cosmid and PAC contig. Genes Chromosomes. Cancer *31*, 228-239.

Beattie,J., Allan,G.J., Lochrie,J.D., and Flint,D.J. (2006). Insulin-like growth factor-binding protein-5 (IGFBP-5): a critical member of the IGF axis. Biochem. J. *395*, 1-19.

Bell,A.C., West,A.G., and Felsenfeld,G. (2001). Insulators and boundaries: versatile regulatory elements in the eukaryotic genome. Science *291*, 447-450.

Bergamaschi,D., Gasco,M., Hiller,L., Sullivan,A., Syed,N., Trigiante,G., Yulug,I., Merlano,M., Numico,G., Comino,A., Attard,M., Reelfs,O., Gusterson,B., Bell,A.K., Heath,V., Tavassoli,M., Farrell,P.J., Smith,P., Lu,X., and Crook,T. (2003). p53 polymorphism influences response in cancer chemotherapy via modulation of p73-dependent apoptosis. Cancer Cell *3*, 387-402.

Bergamaschi,D., Samuels,Y., Sullivan,A., Zvelebil,M., Breyssens,H., Bisso,A., Del Sal,G., Syed,N., Smith,P., Gasco,M., Crook,T., and Lu,X. (2006). iASPP preferentially binds p53 proline-rich region and modulates apoptotic function of codon 72-polymorphic p53. Nat. Genet. *38*, 1133-1141.

Berger,S.L. (2001). An embarrassment of niches: the many covalent modifications of histones in transcriptional regulation. Oncogene *20*, 3007-3013.

Bergholtz,S., Andersen,T.O., Andersson,K.B., Borrebaek,J., Luscher,B., and Gabrielsen,O.S. (2001). The highly conserved DNA-binding domains of A-, B- and c-Myb differ with respect to DNA-binding, phosphorylation and redox properties. Nucleic Acids Res. *29*, 3546-3556.

Berman,D.M., Wang,Y., Liu,Z., Dong,Q., Burke,L.A., Liotta,L.A., Fisher,R., and Wu,X. (2004). A functional polymorphism in RGS6 modulates the risk of bladder cancer. Cancer Res. *64*, 6820-6826.

Bernstein,M.L., Leclerc,J.M., Bunin,G., Brisson,L., Robison,L., Shuster,J., Byrne,T., Gregory,D., Hill,G., Dougherty,G., and . (1992). A population-based study of neuroblastoma incidence, survival, and mortality in North America. J. Clin. Oncol. *10*, 323-329.

Bessa,M., Saville,M.K., and Watson,R.J. (2001). Inhibition of cyclin A/Cdk2 phosphorylation impairs B-Myb transactivation function without affecting interactions with DNA or the CBP coactivator. Oncogene *20*, 3376-3386.

Bies,J., Hoffman,B., Amanullah,A., Giese,T., and Wolff,L. (1996). B-Myb prevents growth arrest associated with terminal differentiation of monocytic cells. Oncogene *12*, 355-363.

Bies,J., Markus,J., and Wolff,L. (2002). Covalent attachment of the SUMO-1 protein to the negative regulatory domain of the c-Myb transcription factor modifies its stability and transactivation capacity. J. Biol. Chem. *277*, 8999-9009.

Bies,J. and Wolff,L. (1997). Oncogenic activation of c-Myb by carboxyl-terminal truncation leads to decreased proteolysis by the ubiquitin-26S proteasome pathway. Oncogene *14*, 203-212.

Bitko,V., Musiyenko,A., Shulyayeva,O., and Barik,S. (2005). Inhibition of respiratory viruses by nasally administered siRNA. Nat. Med. *11*, 50-55.

Bollen,M. (2001). Combinatorial control of protein phosphatase-1. Trends Biochem. Sci. *26*, 426-431.

Boncinelli,E. (1997). Homeobox genes and disease. Curr. Opin. Genet. Dev. *7*, 331-337.

Bond,G.L., Hu,W., Bond,E.E., Robins,H., Lutzker,S.G., Arva,N.C., Bargonetti,J., Bartel,F., Taubert,H., Wuerl,P., Onel,K., Yip,L., Hwang,S.J., Strong,L.C., Lozano,G., and Levine,A.J. (2004). A single nucleotide polymorphism in the MDM2 promoter attenuates the p53 tumor suppressor pathway and accelerates tumor formation in humans. Cell *119*, 591-602.

Boon,K., Caron,H.N., van Asperen,R., Valentijn,L., Hermus,M.C., van Sluis,P., Roobeek,I., Weis,I., Voute,P.A., Schwab,M., and Versteeg,R. (2001). N-myc enhances the expression of a large set of genes functioning in ribosome biogenesis and protein synthesis. EMBO J. *20*, 1383-1393.

Bottini,N., Musumeci,L., Alonso,A., Rahmouni,S., Nika,K., Rostamkhani,M., MacMurray,J., Meloni,G.F., Lucarelli,P., Pellecchia,M., Eisenbarth,G.S.,

Comings,D., and Mustelin,T. (2004). A functional variant of lymphoid tyrosine phosphatase is associated with type I diabetes. Nat. Genet. *36*, 337-338.

Bown,N., Cotterill,S., Lastowska,M., O'Neill,S., Pearson,A.D., Plantaz,D., Meddeb,M., Danglot,G., Brinkschmidt,C., Christiansen,H., Laureys,G., Speleman,F., Nicholson,J., Bernheim,A., Betts,D.R., Vandesompele,J., and Van Roy,N. (1999). Gain of chromosome arm 17q and adverse outcome in patients with neuroblastoma. N. Engl. J. Med. *340*, 1954-1961.

Brandt,T.L., Fraser,D.J., Leal,S., Halandras,P.M., Kroll,A.R., and Kroll,D.J. (1997). c-Myb trans-activates the human DNA topoisomerase IIalpha gene promoter. J. Biol. Chem. *272*, 6278-6284.

Brignole,C., Pastorino,F., Marimpietri,D., Pagnan,G., Pistorio,A., Allen,T.M., Pistoia,V., and Ponzoni,M. (2004). Immune cell-mediated antitumor activities of GD2-targeted liposomal c-myb antisense oligonucleotides containing CpG motifs. J. Natl. Cancer Inst. *96*, 1171-1180.

Brodeur,G.M. (2003). Neuroblastoma: biological insights into a clinical enigma. Nat. Rev. Cancer *3*, 203-216.

Brodeur,G.M., Hayes,F.A., Green,A.A., Casper,J.T., Wasson,J., Wallach,S., and Seeger,R.C. (1987). Consistent N-myc copy number in simultaneous or consecutive neuroblastoma samples from sixty individual patients. Cancer Res. *47*, 4248-4253.

Brodeur,G.M., Maris,J.M., Yamashiro,D.J., Hogarty,M.D., and White,P.S. (1997). Biology and genetics of human neuroblastomas. J. Pediatr. Hematol. Oncol. *19*, 93-101.

Brodeur,G.M., Seeger,R.C., Schwab,M., Varmus,H.E., and Bishop,J.M. (1984). Amplification of N-myc in untreated human neuroblastomas correlates with advanced disease stage. Science *224*, 1121-1124.

Bruno,M.D., Whitsett,J.A., Ross,G.F., and Korfhagen,T.R. (1999). Transcriptional regulation of the murine surfactant protein-A gene by B-Myb. J. Biol. Chem. *274*, 27523-27528.

Burgess,T.L., Fisher,E.F., Ross,S.L., Bready,J.V., Qian,Y.X., Bayewitch,L.A., Cohen,A.M., Herrera,C.J., Hu,S.S., Kramer,T.B., and . (1995). The antiproliferative activity of c-myb and c-myc antisense oligonucleotides in smooth muscle cells is caused by a nonantisense mechanism. Proc. Natl. Acad. Sci. U. S. A *92*, 4051-4055.

Carmeliet,P. (2000). Mechanisms of angiogenesis and arteriogenesis. Nat. Med. *6*, 389-395.

Caron,H., van Sluis,P., Van Roy,N., de Kraker,J., Speleman,F., Voute,P.A., Westerveld,A., Slater,R., and Versteeg,R. (1994). Recurrent 1;17 translocations in human neuroblastoma reveal nonhomologous mitotic recombination during the S/G2 phase as a novel mechanism for loss of heterozygosity. Am. J. Hum. Genet. *55*, 341-347.

Cartegni,L., Chew,S.L., and Krainer,A.R. (2002). Listening to silence and understanding nonsense: exonic mutations that affect splicing. Nat. Rev. Genet. *3*, 285-298.

Casciano,I., Mazzocco,K., Boni,L., Pagnan,G., Banelli,B., Allemanni,G., Ponzoni,M., Tonini,G.P., and Romani,M. (2002). Expression of DeltaNp73 is a molecular marker for adverse outcome in neuroblastoma patients. Cell Death. Differ. *9*, 246-251.

Castle,V.P., Heidelberger,K.P., Bromberg,J., Ou,X., Dole,M., and Nunez,G. (1993). Expression of the apoptosis-suppressing protein bcl-2, in neuroblastoma is associated with unfavorable histology and N-myc amplification. Am. J. Pathol. *143*, 1543-1550.

Catchpole,S., Tavner,F., Le Cam,L., Sardet,C., and Watson,R.J. (2002). A B-myb promoter corepressor site facilitates in vivo occupation of the adjacent E2F site by p107 x E2F and p130 x E2F complexes. J. Biol. Chem. *277*, 39015-39024.

Cervellera,M., Raschella,G., Santilli,G., Tanno,B., Ventura,A., Mancini,C., Sevignani,C., Calabretta,B., and Sala,A. (2000). Direct transactivation of the anti-apoptotic gene apolipoprotein J (clusterin) by B-MYB. J. Biol. Chem. *275*, 21055-21060.

Cervellera,M.N. and Sala,A. (2000). Poly(ADP-ribose) polymerase is a B-MYB coactivator. J. Biol. Chem. *275*, 10692-10696.

Charrasse,S., Carena,I., Brondani,V., Klempnauer,K.H., and Ferrari,S. (2000). Degradation of B-Myb by ubiquitin-mediated proteolysis: involvement of the Cdc34-SCF(p45Skp2) pathway. Oncogene *19*, 2986-2995.

Chen,J., Gammon,M.D., Chan,W., Palomeque,C., Wetmur,J.G., Kabat,G.C., Teitelbaum,S.L., Britton,J.A., Terry,M.B., Neugut,A.I., and Santella,R.M. (2005). One-carbon metabolism, MTHFR polymorphisms, and risk of breast cancer. Cancer Res. *65*, 1606-1614.

Chin,D., Boyle,G.M., Kane,A.J., Theile,D.R., Hayward,N.K., Parson,P.G., and Coman,W.B. (2005). Invasion and metastasis markers in cancers. Br. J. Plast. Surg. *58*, 466-474.

Cho,Y., Gorina,S., Jeffrey,P.D., and Pavletich,N.P. (1994). Crystal structure of a p53 tumor suppressor-DNA complex: understanding tumorigenic mutations. Science *265*, 346-355.

Choi,J., Costa,M.L., Mermelstein,C.S., Chagas,C., Holtzer,S., and Holtzer,H. (1990). MyoD converts primary dermal fibroblasts, chondroblasts, smooth muscle, and retinal pigmented epithelial cells into striated mononucleated myoblasts and multinucleated myotubes. Proc. Natl. Acad. Sci. U. S. A *87*, 7988-7992.

Chong,J.A., Tapia-Ramirez,J., Kim,S., Toledo-Aral,J.J., Zheng,Y., Boutros,M.C., Altshuller,Y.M., Frohman,M.A., Kraner,S.D., and Mandel,G. (1995). REST: a mammalian silencer protein that restricts sodium channel gene expression to neurons. Cell *80*, 949-957.

Cicchillitti,L., Jimenez,S.A., Sala,A., and Saitta,B. (2004). B-Myb acts as a repressor of human COL1A1 collagen gene expression by interacting with Sp1 and CBF factors in scleroderma fibroblasts. Biochem. J. *378*, 609-616.

Ciechanover,A. (1998). The ubiquitin-proteasome pathway: on protein death and cell life. EMBO J. *17*, 7151-7160.

Cirillo,L.A., Lin,F.R., Cuesta,I., Friedman,D., Jarnik,M., and Zaret,K.S. (2002). Opening of compacted chromatin by early developmental transcription factors HNF3 (FoxA) and GATA-4. Mol. Cell *9*, 279-289.

Clagett-Dame,M., McNeill,E.M., and Muley,P.D. (2006). Role of all-trans retinoic acid in neurite outgrowth and axonal elongation. J. Neurobiol. *66*, 739-756.

Cohn,S.L., Salwen,H., Quasney,M.W., Ikegaki,N., Cowan,J.M., Herst,C.V., Kennett,R.H., Rosen,S.T., DiGiuseppe,J.A., and Brodeur,G.M. (1990). Prolonged N-myc protein half-life in a neuroblastoma cell line lacking N-myc amplification. Oncogene *5*, 1821-1827.

Conaway,J.W., Florens,L., Sato,S., Tomomori-Sato,C., Parmely,T.J., Yao,T., Swanson,S.K., Banks,C.A., Washburn,M.P., and Conaway,R.C. (2005). The mammalian Mediator complex. FEBS Lett. *579*, 904-908.

Cooke,M.S., Evans,M.D., Dizdaroglu,M., and Lunec,J. (2003). Oxidative DNA damage: mechanisms, mutation, and disease. FASEB J. *17*, 1195-1214.

Corvi,R., Amler,L.C., Savelyeva,L., Gehring,M., and Schwab,M. (1994). MYCN is retained in single copy at chromosome 2 band p23-24 during amplification in human neuroblastoma cells. Proc. Natl. Acad. Sci. U. S. A *91*, 5523-5527.

Danielian,P.S., White,R., Lees,J.A., and Parker,M.G. (1992). Identification of a conserved region required for hormone dependent transcriptional activation by steroid hormone receptors. EMBO J. *11*, 1025-1033.

Davidson,C.J., Tirouvanziam,R., Herzenberg,L.A., and Lipsick,J.S. (2005). Functional evolution of the vertebrate Myb gene family: B-Myb, but neither A-Myb nor c-Myb, complements Drosophila Myb in hemocytes. Genetics *169*, 215-229.

De Bernardi,B., Nicolas,B., Boni,L., Indolfi,P., Carli,M., Cordero,D.M., Donfrancesco,A., Pession,A., Provenzi,M., di Cataldo,A., Rizzo,A., Tonini,G.P., Dallorso,S., Conte,M., Gambini,C., Garaventa,A., Bonetti,F., Zanazzo,A., D'Angelo,P., and Bruzzi,P. (2003). Disseminated neuroblastoma in children older than one year at diagnosis: comparable results with three consecutive high-dose protocols adopted by the Italian Co-Operative Group for Neuroblastoma. J. Clin. Oncol. *21*, 1592-1601.

de Caestecker,M.P., Yahata,T., Wang,D., Parks,W.T., Huang,S., Hill,C.S., Shioda,T., Roberts,A.B., and Lechleider,R.J. (2000). The Smad4 activation domain (SAD) is a proline-rich, p300-dependent transcriptional activation domain. J. Biol. Chem. *275*, 2115-2122.

De Falco,G., Bagella,L., Claudio,P.P., De Luca,A., Fu,Y., Calabretta,B., Sala,A., and Giordano,A. (2000). Physical interaction between CDK9 and B-Myb results in suppression of B-Myb gene autoregulation. Oncogene *19*, 373-379.

De Gobbi,M., Viprakasit,V., Hughes,J.R., Fisher,C., Buckle,V.J., Ayyub,H., Gibbons,R.J., Vernimmen,D., Yoshinaga,Y., de Jong,P., Cheng,J.F., Rubin,E.M., Wood,W.G., Bowden,D., and Higgs,D.R. (2006). A regulatory SNP causes a human genetic disease by creating a new transcriptional promoter. Science *312*, 1215-1217.

de Jong,M.M., Nolte,I.M., te Meerman,G.J., van der Graaf,W.T., Oosterwijk,J.C., Kleibeuker,J.H., Schaapveld,M., and de Vries,E.G. (2002). Genes other than BRCA1 and BRCA2 involved in breast cancer susceptibility. J. Med. Genet. *39*, 225-242.

de Kraker,J., Hoefnagel,C.A., Caron,H., Valdes Olmos,R.A., Zsiros,J., Heij,H.A., and Voute,P.A. (1995). First line targeted radiotherapy, a new concept in the treatment of advanced stage neuroblastoma. Eur. J. Cancer *31A*, 600-602.

DeGregori,J., Kowalik,T., and Nevins,J.R. (1995). Cellular targets for activation by the E2F1 transcription factor include DNA synthesis- and G1/S-regulatory genes. Mol. Cell Biol. *15*, 4215-4224.

DeRocco,S.E., Iozzo,R., Ma,X.P., Schwarting,R., Peterson,D., and Calabretta,B. (1997). Ectopic expression of A-myb in transgenic mice causes follicular hyperplasia and enhanced B lymphocyte proliferation. Proc. Natl. Acad. Sci. U. S. A *94*, 3240-3244.

Dimri,G.P. (2005). What has senescence got to do with cancer? Cancer Cell *7*, 505-512.

Ding,H.F., Lin,Y.L., McGill,G., Juo,P., Zhu,H., Blenis,J., Yuan,J., and Fisher,D.E. (2000). Essential role for caspase-8 in transcription-independent apoptosis triggered by p53. J. Biol. Chem. *275*, 38905-38911.

Dorsky,R.I., Moon,R.T., and Raible,D.W. (2000). Environmental signals and cell fate specification in premigratory neural crest. Bioessays *22*, 708-716.

Dumont,P., Leu,J.I., Della,P.A., III, George,D.L., and Murphy,M. (2003). The codon 72 polymorphic variants of p53 have markedly different apoptotic potential. Nat. Genet. *33*, 357-365.

Egeblad,M. and Werb,Z. (2002). New functions for the matrix metalloproteinases in cancer progression. Nat. Rev. Cancer *2*, 161-174.

Ekholm,S.V. and Reed,S.I. (2000). Regulation of G(1) cyclin-dependent kinases in the mammalian cell cycle. Curr. Opin. Cell Biol. *12*, 676-684.

El Badry,O.M., Helman,L.J., Chatten,J., Steinberg,S.M., Evans,A.E., and Israel,M.A. (1991). Insulin-like growth factor II-mediated proliferation of human neuroblastoma. J. Clin. Invest *87*, 648-657.

El Badry,O.M., Romanus,J.A., Helman,L.J., Cooper,M.J., Rechler,M.M., and Israel,M.A. (1989). Autonomous growth of a human neuroblastoma cell line is mediated by insulin-like growth factor II. J. Clin. Invest *84*, 829-839.

Elledge,S.J. (1996). Cell cycle checkpoints: preventing an identity crisis. Science *274*, 1664-1672.

Engle,L.J., Simpson,C.L., and Landers,J.E. (2006). Using high-throughput SNP technologies to study cancer. Oncogene *25*, 1594-1601.

Fan,W., Trifiletti,R., Cooper,T., and Norris,J.S. (1992). Cloning of a mu-class glutathione S-transferase gene and identification of the glucocorticoid regulatory domains in its 5' flanking sequence. Proc. Natl. Acad. Sci. U. S. A *89*, 6104-6108.

Ferrao,P., MacMillan,E.M., Ashman,L.K., and Gonda,T.J. (1995). Enforced expression of full length c-Myb leads to density-dependent transformation of murine haemopoietic cells. Oncogene *11*, 1631-1638.

Ferrara,N. and Kerbel,R.S. (2005). Angiogenesis as a therapeutic target. Nature *438*, 967-974.

Fields,S. and Jang,S.K. (1990). Presence of a potent transcription activating sequence in the p53 protein. Science *249*, 1046-1049.

Filipowicz,W. (2005). RNAi: the nuts and bolts of the RISC machine. Cell *122*, 17-20.

Forozan,F., Mahlamaki,E.H., Monni,O., Chen,Y., Veldman,R., Jiang,Y., Gooden,G.C., Ethier,S.P., Kallioniemi,A., and Kallioniemi,O.P. (2000). Comparative genomic hybridization analysis of 38 breast cancer cell lines: a basis for interpreting complementary DNA microarray data. Cancer Res. *60*, 4519-4525.

Fuhrmann,G., Rosenberger,G., Grusch,M., Klein,N., Hofmann,J., and Krupitza,G. (1999). The MYC dualism in growth and death. Mutat. Res. *437*, 205-217.

Fuks,F. (2005). DNA methylation and histone modifications: teaming up to silence genes. Curr. Opin. Genet. Dev. *15*, 490-495.

Gallego,M., Kang,H., and Virshup,D.M. (2006). Protein phosphatase 1 regulates the stability of the circadian protein PER2. Biochem. J. *399*, 169-175.

Garcia,P., Berlanga,O., Watson,R., and Frampton,J. (2005). Generation of a conditional allele of the B-myb gene. Genesis. *43*, 189-195.

Garcia,P. and Frampton,J. (2006). The transcription factor B-Myb is essential for S-phase progression and genomic stability in diploid and polyploid megakaryocytes. J. Cell Sci. *119*, 1483-1493.

Garriga,J. and Grana,X. (2004). Cellular control of gene expression by T-type cyclin/CDK9 complexes. Gene *337*, 15-23.

Gavine,P.R., Neil,J.C., and Crouch,D.H. (1999). Protein stabilization: a common consequence of mutations in independently derived v-Myc alleles. Oncogene *18*, 7552-7558.

Gavioli,R., Frisan,T., Vertuani,S., Bornkamm,G.W., and Masucci,M.G. (2001). c-myc overexpression activates alternative pathways for intracellular proteolysis in lymphoma cells. Nat. Cell Biol. *3*, 283-288.

Gill,G., Pascal,E., Tseng,Z.H., and Tjian,R. (1994). A glutamine-rich hydrophobic patch in transcription factor Sp1 contacts the dTAFII110 component of the Drosophila TFIID complex and mediates transcriptional activation. Proc. Natl. Acad. Sci. U. S. A *91*, 192-196.

Golay,J., Capucci,A., Arsura,M., Castellano,M., Rizzo,V., and Introna,M. (1991). Expression of c-myb and B-myb, but not A-myb, correlates with proliferation in human hematopoietic cells. Blood *77*, 149-158.

Goldschneider,D., Horvilleur,E., Plassa,L.F., Guillaud-Bataille,M., Million,K., Wittmer-Dupret,E., Danglot,G., de The,H., Benard,J., May,E., and Douc-Rasy,S. (2006). Expression of C-terminal deleted p53 isoforms in neuroblastoma. Nucleic Acids Res. *34*, 5603-5612.

Grassilli,E., Salomoni,P., Perrotti,D., Franceschi,C., and Calabretta,B. (1999). Resistance to apoptosis in CTLL-2 cells overexpressing B-Myb is associated with B-Myb-dependent bcl-2 induction. Cancer Res. *59*, 2451-2456.

Graziano,F., Kawakami,K., Ruzzo,A., Watanabe,G., Santini,D., Pizzagalli,F., Bisonni,R., Mari,D., Floriani,I., Catalano,V., Silva,R., Tonini,G., Torri,V., Giustini,L., and Magnani,M. (2006). Methylenetetrahydrofolate reductase 677C/T gene polymorphism, gastric cancer susceptibility and genomic DNA hypomethylation in an at-risk Italian population. Int. J. Cancer *118*, 628-632.

Green,D.R. and Kroemer,G. (2005). Pharmacological manipulation of cell death: clinical applications in sight? J. Clin. Invest *115*, 2610-2617.

Gronroos,E., Hellman,U., Heldin,C.H., and Ericsson,J. (2002). Control of Smad7 stability by competition between acetylation and ubiquitination. Mol. Cell *10*, 483-493.

Gu,J., Wu,X., Dong,Q., Romeo,M.J., Lin,X., Gutkind,J.S., and Berman,D.M. (2006). A nonsynonymous single-nucleotide polymorphism in the PDZ-Rho guanine nucleotide exchange factor (Ser1416Gly) modulates the risk of lung cancer in Mexican Americans. Cancer *106*, 2716-2724.

Guo,C., White,P.S., Weiss,M.J., Hogarty,M.D., Thompson,P.M., Stram,D.O., Gerbing,R., Matthay,K.K., Seeger,R.C., Brodeur,G.M., and Maris,J.M. (1999). Allelic deletion at 11q23 is common in MYCN single copy neuroblastomas. Oncogene *18*, 4948-4957.

Hampsey,M. and Reinberg,D. (2001). RNA Polymerase II Holoenzyme and Transcription Factors. Encyclopedia of Life Sciences.

Hanada,N., Lo,H.W., Day,C.P., Pan,Y., Nakajima,Y., and Hung,M.C. (2006). Co-regulation of B-Myb expression by E2F1 and EGF receptor. Mol. Carcinog. *45*, 10-17.

Hanahan,D. and Weinberg,R.A. (2000). The hallmarks of cancer. Cell *100*, 57-70.

Harper,J.W. and Elledge,S.J. (1999). Skipping into the E2F1-destruction pathway. Nat. Cell Biol. *1*, E5-E7.

Haupt,S., Berger,M., Goldberg,Z., and Haupt,Y. (2003). Apoptosis - the p53 network. J. Cell Sci. *116*, 4077-4085.

Hayashi,Y., Kanda,N., Inaba,T., Hanada,R., Nagahara,N., Muchi,H., and Yamamoto,K. (1989). Cytogenetic findings and prognosis in neuroblastoma with emphasis on marker chromosome 1. Cancer *63*, 126-132.

He,J., Xu,J., Xu,X.X., and Hall,R.A. (2003). Cell cycle-dependent phosphorylation of Disabled-2 by cdc2. Oncogene *22*, 4524-4530.

Hemminki,K. and Shields,P.G. (2002). Skilled use of DNA polymorphisms as a tool for polygenic cancers. Carcinogenesis *23*, 379-380.

Hempstead,B.L. (2002). The many faces of p75NTR. Curr. Opin. Neurobiol. *12*, 260-267.

Henry,M.C., Tashjian,D.B., and Breuer,C.K. (2005). Neuroblastoma update. Curr. Opin. Oncol. *17*, 19-23.

Hibi,K., Liu,Q., Beaudry,G.A., Madden,S.L., Westra,W.H., Wehage,S.L., Yang,S.C., Heitmiller,R.F., Bertelsen,A.H., Sidransky,D., and Jen,J. (1998). Serial analysis of gene expression in non-small cell lung cancer. Cancer Res. *58*, 5690-5694.

Hirai,M., Yoshida,S., Kashiwagi,H., Kawamura,T., Ishikawa,T., Kaneko,M., Ohkawa,H., Nakagawara,A., Miwa,M., and Uchida,K. (1999). 1q23 gain is associated with progressive neuroblastoma resistant to aggressive treatment. Genes Chromosomes. Cancer *25*, 261-269.

Ho,R., Eggert,A., Hishiki,T., Minturn,J.E., Ikegaki,N., Foster,P., Camoratto,A.M., Evans,A.E., and Brodeur,G.M. (2002). Resistance to chemotherapy mediated by TrkB in neuroblastomas. Cancer Res. *62*, 6462-6466.

Hofmann,C.S., Sullivan,C.P., Jiang,H.Y., Stone,P.J., Toselli,P., Reis,E.D., Chereshnev,I., Schreiber,B.M., and Sonenshein,G.E. (2004). B-Myb represses vascular smooth muscle cell collagen gene expression and inhibits neointima formation after arterial injury. Arterioscler. Thromb. Vasc. Biol. *24*, 1608-1613.

Hofmann,C.S., Wang,X., Sullivan,C.P., Toselli,P., Stone,P.J., McLean,S.E., Mecham,R.P., Schreiber,B.M., and Sonenshein,G.E. (2005). B-Myb represses elastin gene expression in aortic smooth muscle cells. J. Biol. Chem. *280*, 7694-7701.

Horikawa,I. and Barrett,J.C. (2003). Transcriptional regulation of the telomerase hTERT gene as a target for cellular and viral oncogenic mechanisms. Carcinogenesis *24*, 1167-1176.

Horiuchi,M., Pratt,R.E., Nakamura,N., and Dzau,V.J. (1993). Distinct nuclear proteins competing for an overlapping sequence of cyclic adenosine monophosphate and negative regulatory elements regulate tissue-specific mouse renin gene expression. J. Clin. Invest *92*, 1805-1811.

Horstmann,S., Ferrari,S., and Klempnauer,K.H. (2000a). An alternatively spliced isoform of B-Myb is a transcriptional inhibitor. Oncogene *19*, 5428-5434.

Horstmann,S., Ferrari,S., and Klempnauer,K.H. (2000b). Regulation of B-Myb activity by cyclin D1. Oncogene *19*, 298-306.

Hoshi,M., Otagiri,N., Shiwaku,H.O., Asakawa,S., Shimizu,N., Kaneko,Y., Ohi,R., Hayashi,Y., and Horii,A. (2000). Detailed deletion mapping of chromosome band 14q32 in human neuroblastoma defines a 1.1-Mb region of common allelic loss. Br. J. Cancer *82*, 1801-1807.

Hoskins,B.E., Thorn,A., Scambler,P.J., and Beales,P.L. (2003). Evaluation of multiplex capillary heteroduplex analysis: a rapid and sensitive mutation screening technique. Hum. Mutat. *22*, 151-157.

Howe,K.M. and Watson,R.J. (1991). Nucleotide preferences in sequence-specific recognition of DNA by c-myb protein. Nucleic Acids Res. *19*, 3913-3919.

Hu,Z., Huo,X., Lu,D., Qian,J., Zhou,J., Chen,Y., Xu,L., Ma,H., Zhu,J., Wei,Q., and Shen,H. (2005). Functional polymorphisms of matrix metalloproteinase-9 are associated with risk of occurrence and metastasis of lung cancer. Clin. Cancer Res. *11*, 5433-5439.

Huang,S., Liu,L.N., Hosoi,H., Dilling,M.B., Shikata,T., and Houghton,P.J. (2001). p53/p21(CIP1) cooperate in enforcing rapamycin-induced G(1) arrest and determine the cellular response to rapamycin. Cancer Res. *61*, 3373-3381.

Humbert,P.O., Verona,R., Trimarchi,J.M., Rogers,C., Dandapani,S., and Lees,J.A. (2000). E2f3 is critical for normal cellular proliferation. Genes Dev. *14*, 690-703.

Hurlin,P.J., Queva,C., Koskinen,P.J., Steingrimsson,E., Ayer,D.E., Copeland,N.G., Jenkins,N.A., and Eisenman,R.N. (1996). Mad3 and Mad4: novel Max-interacting transcriptional repressors that suppress c-myc dependent transformation and are expressed during neural and epidermal differentiation. EMBO J. *15*, 2030.

Imyanitov,E.N., Togo,A.V., and Hanson,K.P. (2004). Searching for cancer-associated gene polymorphisms: promises and obstacles. Cancer Lett. *204*, 3-14.

Introna,M. and Golay,J. (1999). How can oncogenic transcription factors cause cancer: a critical review of the myb story. Leukemia *13*, 1301-1306.

Islam,A., Kageyama,H., Takada,N., Kawamoto,T., Takayasu,H., Isogai,E., Ohira,M., Hashizume,K., Kobayashi,H., Kaneko,Y., and Nakagawara,A. (2000).

High expression of Survivin, mapped to 17q25, is significantly associated with poor prognostic factors and promotes cell survival in human neuroblastoma. Oncogene *19*, 617-623.

Ito,M. (2005). Conservation and diversification of three-repeat Myb transcription factors in plants. J. Plant Res. *118*, 61-69.

Iwai,N., Kitajima,K., Sakai,K., Kimura,T., and Nakano,T. (2001). Alteration of cell adhesion and cell cycle properties of ES cells by an inducible dominant interfering Myb mutant. Oncogene *20*, 1425-1434.

Iwakuma,T., Lozano,G., and Flores,E.R. (2005). Li-Fraumeni syndrome: a p53 family affair. Cell Cycle *4*, 865-867.

Izzi,L. and Attisano,L. (2004). Regulation of the TGFbeta signalling pathway by ubiquitin-mediated degradation. Oncogene *23*, 2071-2078.

Jackson,S.P. (2001). Detecting, signalling and repairing DNA double-strand breaks. Biochem. Soc. Trans. *29*, 655-661.

Jacobs,W.B., Walsh,G.S., and Miller,F.D. (2004). Neuronal survival and p73/p63/p53: a family affair. Neuroscientist. *10*, 443-455.

Jepsen,K. and Rosenfeld,M.G. (2002). Biological roles and mechanistic actions of co-repressor complexes. J. Cell Sci. *115*, 689-698.

Joaquin,M., Bessa,M., Saville,M.K., and Watson,R.J. (2002). B-Myb overcomes a p107-mediated cell proliferation block by interacting with an N-terminal domain of p107. Oncogene *21*, 7923-7932.

Joaquin,M. and Watson,R.J. (2003a). Cell cycle regulation by the B-Myb transcription factor. Cell Mol. Life Sci. *60*, 2389-2401.

Joaquin,M. and Watson,R.J. (2003b). The cell cycle-regulated B-Myb transcription factor overcomes cyclin-dependent kinase inhibitory activity of p57(KIP2) by interacting with its cyclin-binding domain. J. Biol. Chem. *278*, 44255-44264.

Johnson,L.R., Johnson,T.K., Desler,M., Luster,T.A., Nowling,T., Lewis,R.E., and Rizzino,A. (2002). Effects of B-Myb on gene transcription: phosphorylation-dependent activity ans acetylation by p300. J. Biol. Chem. *277*, 4088-4097.

Johnson,T.K., Schweppe,R.E., Septer,J., and Lewis,R.E. (1999). Phosphorylation of B-Myb regulates its transactivation potential and DNA binding. J. Biol. Chem. *274*, 36741-36749.

Kaghad,M., Bonnet,H., Yang,A., Creancier,L., Biscan,J.C., Valent,A., Minty,A., Chalon,P., Lelias,J.M., Dumont,X., Ferrara,P., McKeon,F., and Caput,D. (1997). Monoallelically expressed gene related to p53 at 1p36, a region frequently deleted in neuroblastoma and other human cancers. Cell *90*, 809-819.

Kalcheim,C. and Burstyn-Cohen,T. (2005). Early stages of neural crest ontogeny: formation and regulation of cell delamination. Int. J. Dev. Biol. *49*, 105-116.

Kamano,H., Burk,B., Noben-Trauth,K., and Klempnauer,K.H. (1995). Differential splicing of the mouse B-myb gene. Oncogene *11*, 2575-2582.

Kamano,H. and Klempnauer,K.H. (1997). B-Myb and cyclin D1 mediate heat shock element dependent activation of the human HSP70 promoter. Oncogene *14*, 1223-1229.

Kaneko,Y., Kanda,N., Maseki,N., Nakachi,K., Takeda,T., Okabe,I., and Sakurai,M. (1990). Current urinary mass screening for catecholamine metabolites at 6 months of age may be detecting only a small portion of high-risk neuroblastomas: a chromosome and N-myc amplification study. J. Clin. Oncol. *8*, 2005-2013.

Kaneko,Y., Kanda,N., Maseki,N., Sakurai,M., Tsuchida,Y., Takeda,T., Okabe,I., and Sakurai,M. (1987). Different karyotypic patterns in early and advanced stage neuroblastomas. Cancer Res. *47*, 311-318.

Kaneko,Y. and Knudson,A.G. (2000). Mechanism and relevance of ploidy in neuroblastoma. Genes Chromosomes. Cancer *29*, 89-95.

Kang,J.H., Rychahou,P.G., Ishola,T.A., Qiao,J., Evers,B.M., and Chung,D.H. (2006). MYCN silencing induces differentiation and apoptosis in human neuroblastoma cells. Biochem. Biophys. Res. Commun. *351*, 192-197.

Kaplan,D.R. and Miller,F.D. (2000). Neurotrophin signal transduction in the nervous system. Curr. Opin. Neurobiol. *10*, 381-391.

Karin,M., Cao,Y., Greten,F.R., and Li,Z.W. (2002). NF-kappaB in cancer: from innocent bystander to major culprit. Nat. Rev. Cancer *2*, 301-310.

Katsuda,S. and Kaji,T. (2003). Atherosclerosis and extracellular matrix. J. Atheroscler. Thromb. *10*, 267-274.

Katzen,A.L., Jackson,J., Harmon,B.P., Fung,S.M., Ramsay,G., and Bishop,J.M. (1998). Drosophila myb is required for the G2/M transition and maintenance of diploidy. Genes Dev. *12*, 831-843.

Kellner,U., Sehested,M., Jensen,P.B., Gieseler,F., and Rudolph,P. (2002). Culprit and victim -- DNA topoisomerase II. Lancet Oncol. *3*, 235-243.

Kingma,P.S. and Whitsett,J.A. (2006). In defense of the lung: surfactant protein A and surfactant protein D. Curr. Opin. Pharmacol. *6*, 277-283.

Knudson,A.G. (2000). Chasing the cancer demon. Annu. Rev. Genet. *34*, 1-19.

Knudson,A.G. (2001). Two genetic hits (more or less) to cancer. Nat. Rev. Cancer *1*, 157-162.

Koepp,D.M., Harper,J.W., and Elledge,S.J. (1999). How the cyclin became a cyclin: regulated proteolysis in the cell cycle. Cell *97*, 431-434.

Kogner,P., Barbany,G., Dominici,C., Castello,M.A., Raschella,G., and Persson,H. (1993). Coexpression of messenger RNA for TRK protooncogene and low affinity

nerve growth factor receptor in neuroblastoma with favorable prognosis. Cancer Res. *53*, 2044-2050.

Kono,S. and Chen,K. (2005). Genetic polymorphisms of methylenetetrahydrofolate reductase and colorectal cancer and adenoma. Cancer Sci. *96*, 535-542.

Krams,M., Hero,B., Berthold,F., Parwaresch,R., Harms,D., and Rudolph,P. (2003). Full-length telomerase reverse transcriptase messenger RNA is an independent prognostic factor in neuroblastoma. Am. J. Pathol. *162*, 1019-1026.

Kypreos,K.E., Marhamati,D.J., and Sonenshein,G.E. (1999). B-Myb represses trans-activation of the Col5A2 collagen promoter indirectly via inhibition of binding of factors interacting with positive elements within the first exon. Matrix Biol. *18*, 275-285.

Kypreos,K.E., Nugent,M.A., and Sonenshein,G.E. (1998). Basic fibroblast growth factor-induced decrease in type I collagen gene transcription is mediated by B-myb. Cell Growth Differ. *9*, 723-730.

Ladomery,M. and Dellaire,G. (2002). Multifunctional zinc finger proteins in development and disease. Ann. Hum. Genet. *66*, 331-342.

Lam,E.W., Morris,J.D., Davies,R., Crook,T., Watson,R.J., and Vousden,K.H. (1994). HPV16 E7 oncoprotein deregulates B-myb expression: correlation with targeting of p107/E2F complexes. EMBO J. *13*, 871-878.

Lam,E.W., Robinson,C., and Watson,R.J. (1992). Characterization and cell cycle-regulated expression of mouse B-myb. Oncogene *7*, 1885-1890.

Lam,E.W. and Watson,R.J. (1993). An E2F-binding site mediates cell-cycle regulated repression of mouse B-myb transcription. EMBO J. *12*, 2705-2713.

Lane,S., Farlie,P., and Watson,R. (1997). B-Myb function can be markedly enhanced by cyclin A-dependent kinase and protein truncation. Oncogene *14*, 2445-2453.

Laney,J.D. and Hochstrasser,M. (1999). Substrate targeting in the ubiquitin system. Cell *97*, 427-430.

Lang,G., Gombert,W.M., and Gould,H.J. (2005). A transcriptional regulatory element in the coding sequence of the human Bcl-2 gene. Immunology *114*, 25-36.

Latonen,L. and Laiho,M. (2005). Cellular UV damage responses--functions of tumor suppressor p53. Biochim. Biophys. Acta *1755*, 71-89.

Lavenius,E., Gestblom,C., Johansson,I., Nanberg,E., and Pahlman,S. (1995). Transfection of TRK-A into human neuroblastoma cells restores their ability to differentiate in response to nerve growth factor. Cell Growth Differ. *6*, 727-736.

Lee,T.I. and Young,R.A. (2000). Transcription of eukaryotic protein-coding genes. Annu. Rev. Genet. *34*, 77-137.

Lemon,B. and Tjian,R. (2000). Orchestrated response: a symphony of transcription factors for gene control. Genes Dev. *14*, 2551-2569.

Levy-Lahad,E., Lahad,A., Eisenberg,S., Dagan,E., Paperna,T., Kasinetz,L., Catane,R., Kaufman,B., Beller,U., Renbaum,P., and Gershoni-Baruch,R. (2001). A single nucleotide polymorphism in the RAD51 gene modifies cancer risk in BRCA2 but not BRCA1 carriers. Proc. Natl. Acad. Sci. U. S. A *98*, 3232-3236.

Ley,R., Ewings,K.E., Hadfield,K., Howes,E., Balmanno,K., and Cook,S.J. (2004). Extracellular signal-regulated kinases 1/2 are serum-stimulated "Bim(EL) kinases" that bind to the BH3-only protein Bim(EL) causing its phosphorylation and turnover. J. Biol. Chem. *279*, 8837-8847.

Li,Q., Peterson,K.R., Fang,X., and Stamatoyannopoulos,G. (2002). Locus control regions. Blood *100*, 3077-3086.

Li,X. and McDonnell,D.P. (2002). The transcription factor B-Myb is maintained in an inhibited state in target cells through its interaction with the nuclear corepressors N-CoR and SMRT. Mol. Cell Biol. *22*, 3663-3673.

Lin,D., Fiscella,M., O'Connor,P.M., Jackman,J., Chen,M., Luo,L.L., Sala,A., Travali,S., Appella,E., and Mercer,W.E. (1994). Constitutive expression of B-myb can bypass p53-induced Waf1/Cip1-mediated G1 arrest. Proc. Natl. Acad. Sci. U. S. A *91*, 10079-10083.

Ling,M.T., Wang,X., Lee,D.T., Tam,P.C., Tsao,S.W., and Wong,Y.C. (2004). Id-1 expression induces androgen-independent prostate cancer cell growth through activation of epidermal growth factor receptor (EGF-R). Carcinogenesis *25*, 517-525.

Lipinski,M., Braham,K., Philip,I., Wiels,J., Philip,T., Goridis,C., Lenoir,G.M., and Tursz,T. (1987). Neuroectoderm-associated antigens on Ewing's sarcoma cell lines. Cancer Res. *47*, 183-187.

Lipsick,J.S., Manak,J., Mitiku,N., Chen,C.K., Fogarty,P., and Guthrie,E. (2001). Functional evolution of the Myb oncogene family. Blood Cells Mol. Dis. *27*, 456-458.

Liu,D.X., Biswas,S.C., and Greene,L.A. (2004). B-myb and C-myb play required roles in neuronal apoptosis evoked by nerve growth factor deprivation and DNA damage. J. Neurosci. *24*, 8720-8725.

Liu,D.X. and Greene,L.A. (2001). Regulation of neuronal survival and death by E2F-dependent gene repression and derepression. Neuron *32*, 425-438.

Liu,N., Lucibello,F.C., Zwicker,J., Engeland,K., and Muller,R. (1996). Cell cycle-regulated repression of B-myb transcription: cooperation of an E2F site with a contiguous corepressor element. Nucleic Acids Res. *24*, 2905-2910.

Lutz,W., Stohr,M., Schurmann,J., Wenzel,A., Lohr,A., and Schwab,M. (1996). Conditional expression of N-myc in human neuroblastoma cells increases expression of alpha-prothymosin and ornithine decarboxylase and accelerates

progression into S-phase early after mitogenic stimulation of quiescent cells. Oncogene *13*, 803-812.

Macaluso,M., Montanari,M., and Giordano,A. (2006). Rb family proteins as modulators of gene expression and new aspects regarding the interaction with chromatin remodeling enzymes. Oncogene *25*, 5263-5267.

MacFarlane,M. (2003). TRAIL-induced signalling and apoptosis. Toxicol. Lett. *139*, 89-97.

Manak,J.R., Mitiku,N., and Lipsick,J.S. (2002). Mutation of the Drosophila homologue of the Myb protooncogene causes genomic instability. Proc. Natl. Acad. Sci. U. S. A *99*, 7438-7443.

Mao,X., Orchard,G., Lillington,D.M., Russell-Jones,R., Young,B.D., and Whittaker,S.J. (2003). Amplification and overexpression of JUNB is associated with primary cutaneous T-cell lymphomas. Blood *101*, 1513-1519.

Marhamati,D.J., Bellas,R.E., Arsura,M., Kypreos,K.E., and Sonenshein,G.E. (1997). A-myb is expressed in bovine vascular smooth muscle cells during the late G1-to-S phase transition and cooperates with c-myc to mediate progression to S phase. Mol. Cell Biol. *17*, 2448-2457.

Marhamati,D.J. and Sonenshein,G.E. (1996). B-Myb expression in vascular smooth muscle cells occurs in a cell cycle-dependent fashion and down-regulates promoter activity of type I collagen genes. J. Biol. Chem. *271*, 3359-3365.

Maris,J.M. and Matthay,K.K. (1999). Molecular biology of neuroblastoma. J. Clin. Oncol. *17*, 2264-2279.

Maris,J.M., Weiss,M.J., Guo,C., Gerbing,R.B., Stram,D.O., White,P.S., Hogarty,M.D., Sulman,E.P., Thompson,P.M., Lukens,J.N., Matthay,K.K., Seeger,R.C., and Brodeur,G.M. (2000). Loss of heterozygosity at 1p36 independently predicts for disease progression but not decreased overall survival probability in neuroblastoma patients: a Children's Cancer Group study. J. Clin. Oncol. *18*, 1888-1899.

Martinez,E. (2002). Multi-protein complexes in eukaryotic gene transcription. Plant Mol. Biol. *50*, 925-947.

Masselink,H., Vastenhouw,N., and Bernards,R. (2001). B-myb rescues ras-induced premature senescence, which requires its transactivation domain. Cancer Lett. *171*, 87-101.

Matsunobu,T., Tanaka,K., Matsumoto,Y., Nakatani,F., Sakimura,R., Hanada,M., Li,X., Oda,Y., Naruse,I., Hoshino,H., Tsuneyoshi,M., Miura,H., and Iwamoto,Y. (2004). The prognostic and therapeutic relevance of p27kip1 in Ewing's family tumors. Clin. Cancer Res. *10*, 1003-1012.

Matthay,K.K. (1995). Neuroblastoma: a clinical challenge and biologic puzzle. CA Cancer J. Clin. *45*, 179-192.

Matthay,K.K., Villablanca,J.G., Seeger,R.C., Stram,D.O., Harris,R.E., Ramsay,N.K., Swift,P., Shimada,H., Black,C.T., Brodeur,G.M., Gerbing,R.B., and Reynolds,C.P. (1999). Treatment of high-risk neuroblastoma with intensive chemotherapy, radiotherapy, autologous bone marrow transplantation, and 13-cis-retinoic acid. Children's Cancer Group. N. Engl. J. Med. *341*, 1165-1173.

May,W.A., Lessnick,S.L., Braun,B.S., Klemsz,M., Lewis,B.C., Lunsford,L.B., Hromas,R., and Denny,C.T. (1993). The Ewing's sarcoma EWS/FLI-1 fusion gene encodes a more potent transcriptional activator and is a more powerful transforming gene than FLI-1. Mol. Cell Biol. *13*, 7393-7398.

McIntosh,P.B., Frenkiel,T.A., Wollborn,U., McCormick,J.E., Klempnauer,K.H., Feeney,J., and Carr,M.D. (1998). Solution structure of the B-Myb DNA-binding domain: a possible role for conformational instability of the protein in DNA binding and control of gene expression. Biochemistry *37*, 9619-9629.

Melino,G., Lu,X., Gasco,M., Crook,T., and Knight,R.A. (2003). Functional regulation of p73 and p63: development and cancer. Trends Biochem. Sci. *28*, 663-670.

Mertens,F., Johansson,B., Hoglund,M., and Mitelman,F. (1997). Chromosomal imbalance maps of malignant solid tumors: a cytogenetic survey of 3185 neoplasms. Cancer Res. *57*, 2765-2780.

Meyer,K.B. (2005). B-Myb as a critical regulator of Bcl-2 in human B cells. Immunology *114*, 23-24.

Meyer,T. and Hart,I.R. (1998). Mechanisms of tumour metastasis. Eur. J. Cancer *34*, 214-221.

Mitchell,P.J. and Tjian,R. (1989). Transcriptional regulation in mammalian cells by sequence-specific DNA binding proteins. Science *245*, 371-378.

Mizuguchi,G., Nakagoshi,H., Nagase,T., Nomura,N., Date,T., Ueno,Y., and Ishii,S. (1990). DNA binding activity and transcriptional activator function of the human B-myb protein compared with c-MYB. J. Biol. Chem. *265*, 9280-9284.

Mo,X., Kowenz-Leutz,E., Xu,H., and Leutz,A. (2004). Ras induces mediator complex exchange on C/EBP beta. Mol. Cell *13*, 241-250.

Moll,U.M., LaQuaglia,M., Benard,J., and Riou,G. (1995). Wild-type p53 protein undergoes cytoplasmic sequestration in undifferentiated neuroblastomas but not in differentiated tumors. Proc. Natl. Acad. Sci. U. S. A *92*, 4407-4411.

Monje,P., Marinissen,M.J., and Gutkind,J.S. (2003). Phosphorylation of the carboxyl-terminal transactivation domain of c-Fos by extracellular signal-regulated kinase mediates the transcriptional activation of AP-1 and cellular transformation induced by platelet-derived growth factor. Mol. Cell Biol. *23*, 7030-7043.

Mora,J., Cheung,N.K., Kushner,B.H., LaQuaglia,M.P., Kramer,K., Fazzari,M., Heller,G., Chen,L., and Gerald,W.L. (2000). Clinical categories of neuroblastoma

are associated with different patterns of loss of heterozygosity on chromosome arm 1p. J. Mol. Diagn. *2*, 37-46.

Mucenski,M.L., McLain,K., Kier,A.B., Swerdlow,S.H., Schreiner,C.M., Miller,T.A., Pietryga,D.W., Scott,W.J., Jr., and Potter,S.S. (1991). A functional c-myb gene is required for normal murine fetal hepatic hematopoiesis. Cell *65*, 677-689.

Muller-Tidow,C., Wang,W., Idos,G.E., Diederichs,S., Yang,R., Readhead,C., Berdel,W.E., Serve,H., Saville,M., Watson,R., and Koeffler,H.P. (2001). Cyclin A1 directly interacts with B-myb and cyclin A1/cdk2 phosphorylate B-myb at functionally important serine and threonine residues: tissue-specific regulation of B-myb function. Blood *97*, 2091-2097.

Nakagawara,A., Arima-Nakagawara,M., Scavarda,N.J., Azar,C.G., Cantor,A.B., and Brodeur,G.M. (1993). Association between high levels of expression of the TRK gene and favorable outcome in human neuroblastoma. N. Engl. J. Med. *328*, 847-854.

Nakagawara,A., Azar,C.G., Scavarda,N.J., and Brodeur,G.M. (1994). Expression and function of TRK-B and BDNF in human neuroblastomas. Mol. Cell Biol. *14*, 759-767.

Nakagawara,A. and Ohira,M. (2004). Comprehensive genomics linking between neural development and cancer: neuroblastoma as a model. Cancer Lett. *204*, 213-224.

Nakagoshi,H., Takemoto,Y., and Ishii,S. (1993). Functional domains of the human B-myb gene product. J. Biol. Chem. *268*, 14161-14167.

Narlikar,G.J., Fan,H.Y., and Kingston,R.E. (2002). Cooperation between complexes that regulate chromatin structure and transcription. Cell *108*, 475-487.

Nebert,D.W. (2002). Transcription factors and cancer: an overview. Toxicology *181-182*, 131-141.

Ness,S.A., Marknell,A., and Graf,T. (1989). The v-myb oncogene product binds to and activates the promyelocyte-specific mim-1 gene. Cell *59*, 1115-1125.

Nicoletti,I., Migliorati,G., Pagliacci,M.C., Grignani,F., and Riccardi,C. (1991). A rapid and simple method for measuring thymocyte apoptosis by propidium iodide staining and flow cytometry. J. Immunol. Methods *139*, 271-279.

Nomura,N., Takahashi,M., Matsui,M., Ishii,S., Date,T., Sasamoto,S., and Ishizaki,R. (1988). Isolation of human cDNA clones of myb-related genes, A-myb and B-myb. Nucleic Acids Res. *16*, 11075-11089.

Oh,I.H. and Reddy,E.P. (1999). The myb gene family in cell growth, differentiation and apoptosis. Oncogene *18*, 3017-3033.

Onwuegbusi,B.A., Aitchison,A., Chin,S.F., Kranjac,T., Mills,I., Huang,Y., Lao-Sirieix,P., Caldas,C., and Fitzgerald,R.C. (2006). Impaired transforming growth

factor beta signalling in Barrett's carcinogenesis due to frequent SMAD4 inactivation. Gut *55*, 764-774.

Orlandi,A., Pucci,S., Ciucci,A., Pichiorri,F., Ferlosio,A., and Spagnoli,L.G. (2005). Modulation of clusterin isoforms is associated with all-trans retinoic acid-induced proliferative arrest and apoptosis of intimal smooth muscle cells. Arterioscler. Thromb. Vasc. Biol. *25*, 348-353.

Osterloh,L., von Eyss,B., Schmit,F., Rein,L., Hubner,D., Samans,B., Hauser,S., and Gaubatz,S. (2007). The human synMuv-like protein LIN-9 is required for transcription of G2/M genes and for entry into mitosis. EMBO J. *26*, 144-157.

Oue,T., Fukuzawa,M., Kusafuka,T., Kohmoto,Y., Imura,K., Nagahara,S., and Okada,A. (1996). In situ detection of DNA fragmentation and expression of bcl-2 in human neuroblastoma: relation to apoptosis and spontaneous regression. J. Pediatr. Surg. *31*, 251-257.

Ozanne,B.W., Spence,H.J., McGarry,L.C., and Hennigan,R.F. (2007). Transcription factors control invasion: AP-1 the first among equals. Oncogene *26*, 1-10.

Pabo,C.O. and Sauer,R.T. (1992). Transcription factors: structural families and principles of DNA recognition. Annu. Rev. Biochem. *61*, 1053-1095.

Pagnan,G. and Sala,A. (2003). DNA damage or growth factor withdrawal does not evoke activation of MYB transcription factors in neuronal cancer cell lines. Neurosci. Lett. *336*, 163-166.

Pan,W.H. and Clawson,G.A. (2006). Antisense applications for biological control. J. Cell Biochem. *98*, 14-35.

Papaioannou,G. and McHugh,K. (2005). Neuroblastoma in childhood: review and radiological findings. Cancer Imaging *5*, 116-127.

Patapoutian,A. and Reichardt,L.F. (2001). Trk receptors: mediators of neurotrophin action. Curr. Opin. Neurobiol. *11*, 272-280.

Patikoglou,G. and Burley,S.K. (1997). Eukaryotic transcription factor-DNA complexes. Annu. Rev. Biophys. Biomol. Struct. *26*, 289-325.

Pelham,H.R. and Bienz,M. (1982). A synthetic heat-shock promoter element confers heat-inducibility on the herpes simplex virus thymidine kinase gene. EMBO J. *1*, 1473-1477.

Petrovas,C., Jeay,S., Lewis,R.E., and Sonenshein,G.E. (2003). B-Myb repressor function is regulated by cyclin A phosphorylation and sequences within the C-terminal domain. Oncogene *22*, 2011-2020.

Pilkinton,M., Sandoval,R., Song,J., Ness,S.A., and Colamonici,O.R. (2006). Mip/LIN-9 regulates the expression of B-MYB, and the induction of cyclin a, cyclin B and CDK1. J. Biol. Chem.

Plantaz,D., Vandesompele,J., Van Roy,N., Lastowska,M., Bown,N., Combaret,V., Favrot,M.C., Delattre,O., Michon,J., Benard,J., Hartmann,O., Nicholson,J.C., Ross,F.M., Brinkschmidt,C., Laureys,G., Caron,H., Matthay,K.K., Feuerstein,B.G., and Speleman,F. (2001). Comparative genomic hybridization (CGH) analysis of stage 4 neuroblastoma reveals high frequency of 11q deletion in tumors lacking MYCN amplification. Int. J. Cancer *91*, 680-686.

Ponder,B.A. (2001). Cancer genetics. Nature *411*, 336-341.

Pritchard,J. and Hickman,J.A. (1994). Why does stage 4s neuroblastoma regress spontaneously? Lancet *344*, 869-870.

Raffaghello,L., Marimpietri,D., Pagnan,G., Pastorino,F., Cosimo,E., Brignole,C., Ponzoni,M., and Montaldo,P.G. (2003). Anti-GD2 monoclonal antibody immunotherapy: a promising strategy in the prevention of neuroblastoma relapse. Cancer Lett. *197*, 205-209.

Raschella,G., Cesi,V., Amendola,R., Negroni,A., Tanno,B., Altavista,P., Tonini,G.P., De Bernardi,B., and Calabretta,B. (1999). Expression of B-myb in neuroblastoma tumors is a poor prognostic factor independent from MYCN amplification. Cancer Res. *59*, 3365-3368.

Raschella,G., Negroni,A., Pucci,S., Amendola,R., Valeri,S., and Calabretta,B. (1996). B-myb transcriptional regulation and mRNA stability during differentiation of neuroblastoma cells. Exp. Cell Res. *222*, 395-399.

Raschella,G., Negroni,A., Sala,A., Pucci,S., Romeo,A., and Calabretta,B. (1995). Requirement of b-myb function for survival and differentiative potential of human neuroblastoma cells. J. Biol. Chem. *270*, 8540-8545.

Raschella,G., Tanno,B., Bonetto,F., Amendola,R., Battista,T., De Luca,A., Giordano,A., and Paggi,M.G. (1997). Retinoblastoma-related protein pRb2/p130 and its binding to the B-myb promoter increase during human neuroblastoma differentiation. J. Cell Biochem. *67*, 297-303.

Raschella,G., Tanno,B., Bonetto,F., Negroni,A., Claudio,P.P., Baldi,A., Amendola,R., Calabretta,B., Giordano,A., and Paggi,M.G. (1998). The RB-related gene Rb2/p130 in neuroblastoma differentiation and in B-myb promoter down-regulation. Cell Death. Differ. *5*, 401-407.

Rayman,J.B., Takahashi,Y., Indjeian,V.B., Dannenberg,J.H., Catchpole,S., Watson,R.J., te,R.H., and Dynlacht,B.D. (2002). E2F mediates cell cycle-dependent transcriptional repression in vivo by recruitment of an HDAC1/mSin3B corepressor complex. Genes Dev. *16*, 933-947.

Reynolds,C.P., Matthay,K.K., Villablanca,J.G., and Maurer,B.J. (2003). Retinoid therapy of high-risk neuroblastoma. Cancer Lett. *197*, 185-192.

Robinson,C., Light,Y., Groves,R., Mann,D., Marias,R., and Watson,R. (1996). Cell-cycle regulation of B-Myb protein expression: specific phosphorylation during the S phase of the cell cycle. Oncogene *12*, 1855-1864.

Rozycka,M., Collins,N., Stratton,M.R., and Wooster,R. (2000). Rapid detection of DNA sequence variants by conformation-sensitive capillary electrophoresis. Genomics *70*, 34-40.

Rushton,J.J., Davis,L.M., Lei,W., Mo,X., Leutz,A., and Ness,S.A. (2003). Distinct changes in gene expression induced by A-Myb, B-Myb and c-Myb proteins. Oncogene *22*, 308-313.

Ruteshouser,E.C. and Huff,V. (2004). Familial Wilms tumor. Am. J. Med. Genet. C. Semin. Med. Genet. *129*, 29-34.

Sadler,T.W. (1985). Langman's Medical Embriology., T.M.Tracy, V.M.Vaughn, and W.Vinck, eds. (Baltimore: Williams & Wilkins).

Sala,A. and Calabretta,B. (1992). Regulation of BALB/c 3T3 fibroblast proliferation by B-myb is accompanied by selective activation of cdc2 and cyclin D1 expression. Proc. Natl. Acad. Sci. U. S. A *89*, 10415-10419.

Sala,A., Casella,I., Bellon,T., Calabretta,B., Watson,R.J., and Peschle,C. (1996a). B-myb promotes S phase and is a downstream target of the negative regulator p107 in human cells. J. Biol. Chem. *271*, 9363-9367.

Sala,A., De Luca,A., Giordano,A., and Peschle,C. (1996b). The retinoblastoma family member p107 binds to B-MYB and suppresses its autoregulatory activity. J. Biol. Chem. *271*, 28738-28740.

Sala,A., Kundu,M., Casella,I., Engelhard,A., Calabretta,B., Grasso,L., Paggi,M.G., Giordano,A., Watson,R.J., Khalili,K., and Peschle,C. (1997). Activation of human B-MYB by cyclins. Proc. Natl. Acad. Sci. U. S. A *94*, 532-536.

Sala,A., Saitta,B., De Luca,P., Cervellera,M.N., Casella,I., Lewis,R.E., Watson,R., and Peschle,C. (1999). B-MYB transactivates its own promoter through SP1-binding sites. Oncogene *18*, 1333-1339.

Sala,A. and Watson,R. (1999). B-Myb protein in cellular proliferation, transcription control, and cancer: latest developments. J. Cell Physiol *179*, 245-250.

Saleh,M., Vaillancourt,J.P., Graham,R.K., Huyck,M., Srinivasula,S.M., Alnemri,E.S., Steinberg,M.H., Nolan,V., Baldwin,C.T., Hotchkiss,R.S., Buchman,T.G., Zehnbauer,B.A., Hayden,M.R., Farrer,L.A., Roy,S., and Nicholson,D.W. (2004). Differential modulation of endotoxin responsiveness by human caspase-12 polymorphisms. Nature *429*, 75-79.

Salghetti,S.E., Kim,S.Y., and Tansey,W.P. (1999). Destruction of Myc by ubiquitin-mediated proteolysis: cancer-associated and transforming mutations stabilize Myc. EMBO J. *18*, 717-726.

Sambrook,J., Fitsch,E.F., and Maniatis,T. (1989). Molecular cloning. A laboratory manual. (New York: Cold Spring Harbor Press).

Sandberg,A.A. and Bridge,J.A. (2000). Updates on cytogenetics and molecular genetics of bone and soft tissue tumors: Ewing sarcoma and peripheral primitive neuroectodermal tumors. Cancer Genet. Cytogenet. *123*, 1-26.

Santilli,G., Aronow,B.J., and Sala,A. (2003). Essential requirement of apolipoprotein J (clusterin) signaling for IkappaB expression and regulation of NF-kappaB activity. J. Biol. Chem. *278*, 38214-38219.

Santilli,G., Cervellera,M.N., Johnson,T.K., Lewis,R.E., Iacobelli,S., and Sala,A. (2001). PARP co-activates B-MYB through enhanced phosphorylation at cyclin/cdk2 sites. Oncogene *20*, 8167-8174.

Santilli,G., Schwab,R., Watson,R., Ebert,C., Aronow,B.J., and Sala,A. (2005). Temperature-dependent modification and activation of B-MYB: implications for cell survival. J. Biol. Chem. *280*, 15628-15634.

Saramaki,O.R., Savinainen,K.J., Nupponen,N.N., Bratt,O., and Visakorpi,T. (2001). Amplification of hypoxia-inducible factor 1alpha gene in prostate cancer. Cancer Genet. Cytogenet. *128*, 31-34.

Satterwhite,D.J., Aakre,M.E., Gorska,A.E., and Moses,H.L. (1994). Inhibition of cell growth by TGF beta 1 is associated with inhibition of B-myb and cyclin A in both BALB/MK and Mv1Lu cells. Cell Growth Differ. *5*, 789-799.

Savelyeva,L., Corvi,R., and Schwab,M. (1994). Translocation involving 1p and 17q is a recurrent genetic alteration of human neuroblastoma cells. Am. J. Hum. Genet. *55*, 334-340.

Saville,M.K. and Watson,R.J. (1998). The cell-cycle regulated transcription factor B-Myb is phosphorylated by cyclin A/Cdk2 at sites that enhance its transactivation properties. Oncogene *17*, 2679-2689.

Sawada,T., Sugimoto,T., Tanaka,T., Kawakatsu,H., Ishii,T., Matsumura,T., and Horii,Y. (1987). Number and cure rate of neuroblastoma cases detected by the mass screening program in Japan: future aspects. Med. Pediatr. Oncol. *15*, 14-17.

Scherer,L.J. and Rossi,J.J. (2003). Approaches for the sequence-specific knockdown of mRNA. Nat. Biotechnol. *21*, 1457-1465.

Schreiber,V., Dantzer,F., Ame,J.C., and de Murcia,G. (2006). Poly(ADP-ribose): novel functions for an old molecule. Nat. Rev. Mol. Cell Biol. *7*, 517-528.

Schubert,S., Horstmann,S., Bartusel,T., and Klempnauer,K.H. (2004). The cooperation of B-Myb with the coactivator p300 is orchestrated by cyclins A and D1. Oncogene *23*, 1392-1404.

Schwab,M., Ellison,J., Busch,M., Rosenau,W., Varmus,H.E., and Bishop,J.M. (1984). Enhanced expression of the human gene N-myc consequent to amplification of DNA may contribute to malignant progression of neuroblastoma. Proc. Natl. Acad. Sci. U. S. A *81*, 4940-4944.

Seeger,R.C., Brodeur,G.M., Sather,H., Dalton,A., Siegel,S.E., Wong,K.Y., and Hammond,D. (1985). Association of multiple copies of the N-myc oncogene with rapid progression of neuroblastomas. N. Engl. J. Med. *313*, 1111-1116.

Shannan,B., Seifert,M., Leskov,K., Willis,J., Boothman,D., Tilgen,W., and Reichrath,J. (2006). Challenge and promise: roles for clusterin in pathogenesis, progression and therapy of cancer. Cell Death. Differ. *13*, 12-19.

Shepard,J.L., Amatruda,J.F., Stern,H.M., Subramanian,A., Finkelstein,D., Ziai,J., Finley,K.R., Pfaff,K.L., Hersey,C., Zhou,Y., Barut,B., Freedman,M., Lee,C., Spitsbergen,J., Neuberg,D., Weber,G., Golub,T.R., Glickman,J.N., Kutok,J.L., Aster,J.C., and Zon,L.I. (2005). A zebrafish bmyb mutation causes genome instability and increased cancer susceptibility. Proc. Natl. Acad. Sci. U. S. A *102*, 13194-13199.

Shimada,H., Ambros,I.M., Dehner,L.P., Hata,J., Joshi,V.V., and Roald,B. (1999). Terminology and morphologic criteria of neuroblastic tumors: recommendations by the International Neuroblastoma Pathology Committee. Cancer *86*, 349-363.

Shohet,J.M., Hicks,M.J., Plon,S.E., Burlingame,S.M., Stuart,S., Chen,S.Y., Brenner,M.K., and Nuchtern,J.G. (2002). Minichromosome maintenance protein MCM7 is a direct target of the MYCN transcription factor in neuroblastoma. Cancer Res. *62*, 1123-1128.

Sitzmann,J., Noben-Trauth,K., Kamano,H., and Klempnauer,K.H. (1996). Expression of B-Myb during mouse embryogenesis. Oncogene *12*, 1889-1894.

Skotheim,R.I., Monni,O., Mousses,S., Fossa,S.D., Kallioniemi,O.P., Lothe,R.A., and Kallioniemi,A. (2002). New insights into testicular germ cell tumorigenesis from gene expression profiling. Cancer Res. *62*, 2359-2364.

Slack,A., Chen,Z., Tonelli,R., Pule,M., Hunt,L., Pession,A., and Shohet,J.M. (2005). The p53 regulatory gene MDM2 is a direct transcriptional target of MYCN in neuroblastoma. Proc. Natl. Acad. Sci. U. S. A *102*, 731-736.

Small,M.B., Hay,N., Schwab,M., and Bishop,J.M. (1987). Neoplastic transformation by the human gene N-myc. Mol. Cell Biol. *7*, 1638-1645.

Song,E., Zhu,P., Lee,S.K., Chowdhury,D., Kussman,S., Dykxhoorn,D.M., Feng,Y., Palliser,D., Weiner,D.B., Shankar,P., Marasco,W.A., and Lieberman,J. (2005). Antibody mediated in vivo delivery of small interfering RNAs via cell-surface receptors. Nat. Biotechnol. *23*, 709-717.

Spitz,R., Hero,B., Ernestus,K., and Berthold,F. (2003). Deletions in chromosome arms 3p and 11q are new prognostic markers in localized and 4s neuroblastoma. Clin. Cancer Res. *9*, 52-58.

Srivastava,V.K. and Busbee,D.L. (2003). Replicative enzymes, DNA polymerase alpha (pol alpha), and in vitro ageing. Exp. Gerontol. *38*, 1285-1297.

Stacey,S.N., Sulem,P., Johannsson,O.T., Helgason,A., Gudmundsson,J., Kostic,J.P., Kristjansson,K., Jonsdottir,T., Sigurdsson,H., Hrafnkelsson,J.,

Johannsson,J., Sveinsson,T., Myrdal,G., Grimsson,H.N., Bergthorsson,J.T., Amundadottir,L.T., Gulcher,J.R., Thorsteinsdottir,U., Kong,A., and Stefansson,K. (2006). The BARD1 Cys557Ser variant and breast cancer risk in Iceland. PLoS. Med. *3*, e217.

Sugihara,E., Kanai,M., Matsui,A., Onodera,M., Schwab,M., and Miwa,M. (2004). Enhanced expression of MYCN leads to centrosome hyperamplification after DNA damage in neuroblastoma cells. Oncogene *23*, 1005-1009.

Sullivan,A., Syed,N., Gasco,M., Bergamaschi,D., Trigiante,G., Attard,M., Hiller,L., Farrell,P.J., Smith,P., Lu,X., and Crook,T. (2004). Polymorphism in wild-type p53 modulates response to chemotherapy in vitro and in vivo. Oncogene *23*, 3328-3337.

Sullivan,K.A., Castle,V.P., Hanash,S.M., and Feldman,E.L. (1995). Insulin-like growth factor II in the pathogenesis of human neuroblastoma. Am. J. Pathol. *147*, 1790-1798.

Suzuki,T., Bogenmann,E., Shimada,H., Stram,D., and Seeger,R.C. (1993). Lack of high-affinity nerve growth factor receptors in aggressive neuroblastomas. J. Natl. Cancer Inst. *85*, 377-384.

Tacconelli,A., Farina,A.R., Cappabianca,L., Desantis,G., Tessitore,A., Vetuschi,A., Sferra,R., Rucci,N., Argenti,B., Screpanti,I., Gulino,A., and Mackay,A.R. (2004). TrkA alternative splicing: a regulated tumor-promoting switch in human neuroblastoma. Cancer Cell *6*, 347-360.

Takahashi,T., Nakagoshi,H., Sarai,A., Nomura,N., Yamamoto,T., and Ishii,S. (1995). Human A-myb gene encodes a transcriptional activator containing the negative regulatory domains. FEBS Lett. *358*, 89-96.

Takahashi,Y., Rayman,J.B., and Dynlacht,B.D. (2000). Analysis of promoter binding by the E2F and pRB families in vivo: distinct E2F proteins mediate activation and repression. Genes Dev. *14*, 804-816.

Takita,J., Yang,H.W., Chen,Y.Y., Hanada,R., Yamamoto,K., Teitz,T., Kidd,V., and Hayashi,Y. (2001). Allelic imbalance on chromosome 2q and alterations of the caspase 8 gene in neuroblastoma. Oncogene *20*, 4424-4432.

Tamm,I., Schriever,F., and Dorken,B. (2001). Apoptosis: implications of basic research for clinical oncology. Lancet Oncol. *2*, 33-42.

Tanaka,Y., Patestos,N.P., Maekawa,T., and Ishii,S. (1999). B-myb is required for inner cell mass formation at an early stage of development. J. Biol. Chem. *274*, 28067-28070.

Tanner,M.M., Grenman,S., Koul,A., Johannsson,O., Meltzer,P., Pejovic,T., Borg,A., and Isola,J.J. (2000). Frequent amplification of chromosomal region 20q12-q13 in ovarian cancer. Clin. Cancer Res. *6*, 1833-1839.

Tanno,B., Negroni,A., Vitali,R., Pirozzoli,M.C., Cesi,V., Mancini,C., Calabretta,B., and Raschella,G. (2002). Expression of insulin-like growth factor-binding protein 5

in neuroblastoma cells is regulated at the transcriptional level by c-Myb and B-Myb via direct and indirect mechanisms. J. Biol. Chem. *277*, 23172-23180.

Tashiro,S., Sumi,T., Uozumi,N., Shimizu,T., and Nakamura,T. (2004). B-Myb-dependent regulation of c-Myc expression by cytosolic phospholipase A2. J. Biol. Chem. *279*, 17715-17722.

Tashiro,S., Takemoto,Y., Handa,H., and Ishii,S. (1995). Cell type-specific trans-activation by the B-myb gene product: requirement of the putative cofactor binding to the C-terminal conserved domain. Oncogene *10*, 1699-1707.

Taub,R., Kirsch,I., Morton,C., Lenoir,G., Swan,D., Tronick,S., Aaronson,S., and Leder,P. (1982). Translocation of the c-myc gene into the immunoglobulin heavy chain locus in human Burkitt lymphoma and murine plasmacytoma cells. Proc. Natl. Acad. Sci. U. S. A *79*, 7837-7841.

Tavner,F., Frampton,J., and Watson,R.J. (2006). Targeting an E2F site in the mouse genome prevents promoter silencing in quiescent and post-mitotic cells. Oncogene.

Teitz,T., Wei,T., Valentine,M.B., Vanin,E.F., Grenet,J., Valentine,V.A., Behm,F.G., Look,A.T., Lahti,J.M., and Kidd,V.J. (2000). Caspase 8 is deleted or silenced preferentially in childhood neuroblastomas with amplification of MYCN. Nat. Med. *6*, 529-535.

Thiele,C.J., Cohen,P.S., and Israel,M.A. (1988). Regulation of c-myb expression in human neuroblastoma cells during retinoic acid-induced differentiation. Mol. Cell Biol. *8*, 1677-1683.

Thompson,P.M., Maris,J.M., Hogarty,M.D., Seeger,R.C., Reynolds,C.P., Brodeur,G.M., and White,P.S. (2001). Homozygous deletion of CDKN2A (p16INK4a/p14ARF) but not within 1p36 or at other tumor suppressor loci in neuroblastoma. Cancer Res. *61*, 679-686.

Tomari,Y., Du,T., Haley,B., Schwarz,D.S., Bennett,R., Cook,H.A., Koppetsch,B.S., Theurkauf,W.E., and Zamore,P.D. (2004). RISC assembly defects in the Drosophila RNAi mutant armitage. Cell *116*, 831-841.

Torelli,G., Venturelli,D., Colo,A., Zanni,C., Selleri,L., Moretti,L., Calabretta,B., and Torelli,U. (1987). Expression of c-myb protooncogene and other cell cycle-related genes in normal and neoplastic human colonic mucosa. Cancer Res. *47*, 5266-5269.

Toscani,A., Mettus,R.V., Coupland,R., Simpkins,H., Litvin,J., Orth,J., Hatton,K.S., and Reddy,E.P. (1997). Arrest of spermatogenesis and defective breast development in mice lacking A-myb. Nature *386*, 713-717.

Trauth,K., Mutschler,B., Jenkins,N.A., Gilbert,D.J., Copeland,N.G., and Klempnauer,K.H. (1994). Mouse A-myb encodes a trans-activator and is expressed in mitotically active cells of the developing central nervous system, adult testis and B lymphocytes. EMBO J. *13*, 5994-6005.

Treier,M., Staszewski,L.M., and Bohmann,D. (1994). Ubiquitin-dependent c-Jun degradation in vivo is mediated by the delta domain. Cell *78*, 787-798.

Tsurumi,C., Ishida,N., Tamura,T., Kakizuka,A., Nishida,E., Okumura,E., Kishimoto,T., Inagaki,M., Okazaki,K., Sagata,N., and . (1995). Degradation of c-Fos by the 26S proteasome is accelerated by c-Jun and multiple protein kinases. Mol. Cell Biol. *15*, 5682-5687.

Tucker,R.P. (2004). Neural crest cells: a model for invasive behavior. Int. J. Biochem. Cell Biol. *36*, 173-177.

Valko,M., Leibfritz,D., Moncol,J., Cronin,M.T., Mazur,M., and Telser,J. (2007). Free radicals and antioxidants in normal physiological functions and human disease. Int. J. Biochem. Cell Biol. *39*, 44-84.

van Dam,H. and Castellazzi,M. (2001). Distinct roles of Jun : Fos and Jun : ATF dimers in oncogenesis. Oncogene *20*, 2453-2464.

van Noesel,M.M. and Versteeg,R. (2004). Pediatric neuroblastomas: genetic and epigenetic 'danse macabre'. Gene *325*, 1-15.

Vandesompele,J., Speleman,F., Van Roy,N., Laureys,G., Brinskchmidt,C., Christiansen,H., Lampert,F., Lastowska,M., Bown,N., Pearson,A., Nicholson,J.C., Ross,F., Combaret,V., Delattre,O., Feuerstein,B.G., and Plantaz,D. (2001). Multicentre analysis of patterns of DNA gains and losses in 204 neuroblastoma tumors: how many genetic subgroups are there? Med. Pediatr. Oncol. *36*, 5-10.

Vogan,K., Bernstein,M., Leclerc,J.M., Brisson,L., Brossard,J., Brodeur,G.M., Pelletier,J., and Gros,P. (1993). Absence of p53 gene mutations in primary neuroblastomas. Cancer Res. *53*, 5269-5273.

Vogt,W. (1995). Oxidation of methionyl residues in proteins: tools, targets, and reversal. Free Radic. Biol. Med. *18*, 93-105.

Wang,Z. and Moult,J. (2001). SNPs, protein structure, and disease. Hum. Mutat. *17*, 263-270.

Wartiovaara,K., Barnabe-Heider,F., Miller,F.D., and Kaplan,D.R. (2002). N-myc promotes survival and induces S-phase entry of postmitotic sympathetic neurons. J. Neurosci. *22*, 815-824.

Wassarman,D.A. and Sauer,F. (2001). TAF(II)250: a transcription toolbox. J. Cell Sci. *114*, 2895-2902.

Watson,R.J., Robinson,C., and Lam,E.W. (1993). Transcription regulation by murine B-myb is distinct from that by c-myb. Nucleic Acids Res. *21*, 267-272.

Weinstein,I.B. (2002). Cancer. Addiction to oncogenes--the Achilles heal of cancer. Science *297*, 63-64.

Weiss,W.A., Aldape,K., Mohapatra,G., Feuerstein,B.G., and Bishop,J.M. (1997). Targeted expression of MYCN causes neuroblastoma in transgenic mice. EMBO J. *16*, 2985-2995.

Wells,J., Boyd,K.E., Fry,C.J., Bartley,S.M., and Farnham,P.J. (2000). Target gene specificity of E2F and pocket protein family members in living cells. Mol. Cell Biol. *20*, 5797-5807.

Wenzel,A. and Schwab,M. (1995). The mycN/max protein complex in neuroblastoma. Short review. Eur. J. Cancer *31A*, 516-519.

Westermann,F. and Schwab,M. (2002). Genetic parameters of neuroblastomas. Cancer Lett. *184*, 127-147.

Westin,E.H., Gallo,R.C., Arya,S.K., Eva,A., Souza,L.M., Baluda,M.A., Aaronson,S.A., and Wong-Staal,F. (1982). Differential expression of the amv gene in human hematopoietic cells. Proc. Natl. Acad. Sci. U. S. A *79*, 2194-2198.

Weston,K. (1999). Reassessing the role of C-MYB in tumorigenesis. Oncogene *18*, 3034-3038.

Woychik,N.A. and Hampsey,M. (2002). The RNA polymerase II machinery: structure illuminates function. Cell *108*, 453-463.

Yaffe,M.B., Schutkowski,M., Shen,M., Zhou,X.Z., Stukenberg,P.T., Rahfeld,J.U., Xu,J., Kuang,J., Kirschner,M.W., Fischer,G., Cantley,L.C., and Lu,K.P. (1997). Sequence-specific and phosphorylation-dependent proline isomerization: a potential mitotic regulatory mechanism. Science *278*, 1957-1960.

Yam,C.H., Fung,T.K., and Poon,R.Y. (2002). Cyclin A in cell cycle control and cancer. Cell Mol. Life Sci. *59*, 1317-1326.

Yamashiro,D.J., Liu,X.G., Lee,C.P., Nakagawara,A., Ikegaki,N., McGregor,L.M., Baylin,S.B., and Brodeur,G.M. (1997). Expression and function of Trk-C in favourable human neuroblastomas. Eur. J. Cancer *33*, 2054-2057.

Yancopoulos,G.D., Davis,S., Gale,N.W., Rudge,J.S., Wiegand,S.J., and Holash,J. (2000). Vascular-specific growth factors and blood vessel formation. Nature *407*, 242-248.

Yang,R., Morosetti,R., and Koeffler,H.P. (1997). Characterization of a second human cyclin A that is highly expressed in testis and in several leukemic cell lines. Cancer Res. *57*, 913-920.

Yanik,G.A., Levine,J.E., Matthay,K.K., Sisson,J.C., Shulkin,B.L., Shapiro,B., Hubers,D., Spalding,S., Braun,T., Ferrara,J.L., and Hutchinson,R.J. (2002). Pilot study of iodine-131-metaiodobenzylguanidine in combination with myeloablative chemotherapy and autologous stem-cell support for the treatment of neuroblastoma. J. Clin. Oncol. *20*, 2142-2149.

Yarden,Y. (2001). The EGFR family and its ligands in human cancer. signalling mechanisms and therapeutic opportunities. Eur. J. Cancer *37 Suppl 4*, S3-S8.

Yin,Y., Stephen,C.W., Luciani,M.G., and Fahraeus,R. (2002). p53 Stability and activity is regulated by Mdm2-mediated induction of alternative p53 translation products. Nat. Cell Biol. *4*, 462-467.

Yu,X., Caltagarone,J., Smith,M.A., and Bowser,R. (2005). DNA damage induces cdk2 protein levels and histone H2B phosphorylation in SH-SY5Y neuroblastoma cells. J. Alzheimers. Dis. *8*, 7-21.

Zhang,X., Kon,T., Wang,H., Li,F., Huang,Q., Rabbani,Z.N., Kirkpatrick,J.P., Vujaskovic,Z., Dewhirst,M.W., and Li,C.Y. (2004). Enhancement of hypoxia-induced tumor cell death in vitro and radiation therapy in vivo by use of small interfering RNA targeted to hypoxia-inducible factor-1alpha. Cancer Res. *64*, 8139-8142.

Zhu,W., Giangrande,P.H., and Nevins,J.R. (2004). E2Fs link the control of G1/S and G2/M transcription. EMBO J. *23*, 4615-4626.

Ziebold,U., Bartsch,O., Marais,R., Ferrari,S., and Klempnauer,K.H. (1997). Phosphorylation and activation of B-Myb by cyclin A-Cdk2. Curr. Biol. *7*, 253-260.

Zondervan,P.E., Wink,J., Alers,J.C., IJzermans,J.N., Schalm,S.W., de Man,R.A., and van Dekken,H. (2000). Molecular cytogenetic evaluation of virus-associated and non-viral hepatocellular carcinoma: analysis of 26 carcinomas and 12 concurrent dysplasias. J. Pathol. *192*, 207-215.

Zwicker,J., Liu,N., Engeland,K., Lucibello,F.C., and Muller,R. (1996). Cell cycle regulation of E2F site occupation in vivo. Science *271*, 1595-1597.

Zylicz,M., King,F.W., and Wawrzynow,A. (2001). Hsp70 interactions with the p53 tumour suppressor protein. EMBO J. *20*, 4634-4638.